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AUSTRALIA

Patents Act 1990

IRAJ GHADIMINEJAD

PROVISIONAL SPECIFICATION

Invention Title:

A low molecular weight, non-proteinaceous compound that inhibits mitogen induced cytokine production

The invention is described in the following statement:

A low molecular weight, non-proteinaceous compound that inhibits mitogen induced cytokine production.

FIELD OF THE INVENTION

The present invention relates to new compounds which inhibit LPS induced production of TNF-α and/or IL-1α. The compound may be isolated from urine of patients suffering from steroid responsive nephrotic syndrome or can be chemically synthesised. The compound has application in the treatment and/or prevention of Gram negative septic shock.

BACKGROUND OF THE INVENTION

Despite immense efforts over the last decade to reduce the mortality from endotoxicosis consequent to sepsis, gram-negative septic shock kills over 150,000 people each year in USA alone.

Endotoxin [also known as lipopolysaccharide (LPS)] is the major constituent of the outer membrane of Gram-negative bacteria. They cause a variety of pathophysiological effects in human and experimental animals. Bacteria release endotoxin when in circulation, causing the release of a number of pro-inflammatory cytokines. It is noteworthy that the use of antibiotics results in bacterial death, and release of endotoxin. This in turn may exacerbate the problem by overwhelming the immune system, leading to shock.

Huge amounts of money have been spent in developing new therapeutic agents, including antibodies against endotoxin, soluble tumour necrosis factor (TNF) receptors, and synthetically produced antagonists of endotoxin. The failure of the anti-endotoxin antibody HA1A trial seems to have been mainly due to the fact that these antibodies were not able to neutralise the toxic effects of endotoxin.

Polymyxin B, a cationic cyclic peptide antibiotic, which inhibits the biological effects of endotoxin through its high affinity binding to lipid A, has also been tested as a potential therapeutic candidate. However its use is limited by its high toxicity. Unfortunately none of these agents have proved particularly beneficial.

The young and geriatrics are more susceptible to bacterial infection with high mortality. These failed trials demonstrate the pressing need to develop effective, bioavailable therapeutics.

Steroid Responsive Nephrotic Syndrome is the most frequent nephrotic disorder in children, where 5-10 gram of serum albumin can be excreted in urine over a 24 hour cycle.

The kidney has been shown to function both as a size and a charge selective barrier (Rennke, 1978; Chang, 1975). The glomerular basement membrane (GBM) and the foot processes of the epithelial cells have been shown to be covered by glycoproteins bearing negative charge (Andrews, 1979; Michael, 1970). The charge selectivity of the glomerulus has been attributed to fixed anionic sites, shown to be essentially heparan sulphate (Kanwar and Farquhar, 1979; Kanwar et al. 1980). In experimental animals, increased permeability to serum

proteins, in particular serum albumin, is associated with the reduction in the number of fixed anionic sites (Caulfield, 1976; Caulfield and Farquhar, 1978).

The aetiology and pathogenesis of Steroid Responsive Nephrotic Syndrome (SRNS) also known as Minimal Change Nephrotic syndrome (MCNS) are unknown. SRNS is the most frequent form of nephrotic syndrome in children with the peak incidence at 3-5 years of age. Patients with SRNS characteristically have normal glomerular filtration with little or no change observed in kidney biopsies under light microscopy (Mallick, 1991; Adamson et al. 1986). However, the fusion of the foot processes are only observed with the use of electron microscopy (Powell, 1976). Focal Segmental Glomerular Sclerosis (FSGS) shares many clinical and pathological features with SRNS (Yoshikawa et al. 1986; Yoshikawa et al. 1982), and its peak incidence occurs in older children and young adults. FSGS is thought to be the end stage renal disease form of SRNS, and patients with FSGS do not often respond to steroid treatment (Schena and Cameron, 1988).
Robson *et al* (Robson et al. 1974) were the first to suggest that loss of charge might be responsible for high albumin excretion and that in SRNS this charge barrier may be reduced. Bohrer *et al* (Bohrer et al. 1978) clearly demonstrated that charge plays an important role in glomerular filtration by infusing rats with neutral, anionic and cationic dextran of various molecular weights, showing that for a given molecular size, the neutral and the positively charged molecules were filtered at a greater rate.

Shalhoub (Shalhoub, 1974), on the basis of the following clinical observations suggested that in SRNS patients, circulating factors resulting from a systemic abnormality in T-cells may play an important role in the pathogenesis of the nephrotic syndrome.

1. Association of the remission of lipoid nephrosis with natural measles
2. Induction of remission by steroids that is prolonged by treatment with cyclophosphamide
3. Occurrence of similar glomerular disorders in Hodgkin and non-Hodgkin's lymphoma diseases.

Wilkinson *et al* (Wilkinson et al. 1989) showed that *in vivo* infusion of plasma from patients with SRNS into rabbits, reduced anionic sites in the GBM with a concomitant increase in proteinuria. Serum from patients with recurrent FSGS in renal allograft also caused proteinuria (Zimmerman and Mann, 1984). As early as 1975, Lagrue *et al* (Lagrue et al. 1975) reported the presence of a vascular permeability factor (VPF) produced by the peripheral blood mononuclear cells (PBMC) from SRNS children stimulated in culture with concanavalin A (Con A). Furthermore, Tanaka *et al* (Tanaka et al. 1992), also showed that supernatants of cultured PBMC from children with SRNS and FSGS, after stimulation with Con A, caused proteinuria and a decrease in the fixed anionic sites in the GBM. A number of groups have investigated this phenomena and the current consensus is that VPF is a heat labile, pepsin-sensitive protein or glycoprotein of approximately 12kDa produced by T lymphocytes (Sobel et al. 1977; Heslan et al. 1986). Tomizawa *et al* (Tomizawa et al. 1985) reported that low molecular size plasma fractions from active SRNS patients had an inhibitory effect on the

production of VPF when added to isolated lymphocytes from patients that were previously shown to produce this permeability factor in culture. However, the production of VPF has been shown to be associated with other nephrotic syndromes such as IgA nephropathy. It was therefore concluded that VPF may not be directly related to the increased glomerular permeability (Bakker et al. 1982).

Studies by Matsumoto *et al* (Matsumoto et al. 1984) have revealed abnormalities in the cellular immune system, followed by evidence illustrating impaired T-lymphocyte colony formation in lipoid nephrosis (Matsumoto et al. 1985). Further evidence for immune abnormalities of lymphocytes (Sasdelli et al. 1980) and the function of T-cells, such as depressed lymphocyte blast formation with some T-cell mitogens (foder et al. 1982) were also provided.

The hypothesis that loss in the regulation of immune response, stimulated by an unknown antigen(s) is responsible for the pathogenesis of SRNS is further supported by the presence of soluble immune response suppressor (SIRS), in the urine and sera of SRNS patients. SIRS are a family of proteins that can be produced by interferon (IFN) or ConA activated suppressor T lymphocytes (Schnaper et al. 1984; Cheng et al. 1989; Schnaper and Aune, 1985; Schnaper and Aune, 1987).

The inventor's initial work on the nephrotic disorder, Steroid Responsive Nephrotic Syndrome concentrated on the purification of a postulated circulatory highly cationic protein (Levin et al. 1989) that was said to neutralise negatively charged heparan sulphate leading to proteinuria. Given the fact that Heparan sulphate is the major glycosaminoglycan coating the glomerular basement membrane, producing an electrostatic charge barrier, the concept of a circulatory highly cationic protein was an attractive one.

The established methodology for the detection of glycosaminoglycans (GAGs) was based on the precipitation of highly anionic GAGs with cationic dyes and detergents. The methodology developed by Whiteman (Whiteman, 1973; Whitemen, 1973) with minor modifications (Levin et al. 1989) using the cationic dye Alcian Blue for the precipitation and quantification of GAGs was used for the detection of this hypothesised cationic protein. A successful purification procedure from the urine of children with SRNS was established where the presence of the cationic protein in the chromatographic fractions was detected using this methodology.

SDS-PAGE analysis of this protein showed two bands, a 50kDa and a 25kDa molecular weight band, under reducing conditions (Fig 1). The amino acid sequencing data from these bands suggested that the purified protein was of immunoglobulin origin (not shown).

During purification of the hypothesised cationic protein, the present inventor discovered that the urine from these patients contained an inhibitor of LPS, demonstrated by utilising the Limulus Amoebocyte Lysate (LAL) assay system.

This factor was named "Nephronin" to reflect the source and the origin of the material.

Table 1 shows a typical endotoxin neutralising experiment performed for Nephronin utilising the LAL assay. This activity was associated with a low molecular weight, non-proteinaceous, heat stable molecule that could also agglutinate Gram-negative bacteria.

Preliminary studies have shown that Nephronin inhibits cytokine production in LPS-stimulated monocytes and fibroblasts. This combination of low molecular mass and heat- and protease-stability suggests that Nephronin could have potential as a therapeutic agent for sepsis.

Lipid A is the most conserved region of LPS and is responsible for virtually all of the toxic activities of LPS. LPS stimulates a range of cells, most notably macrophages (MØ), to synthesise cytokines including interleukin-1α (IL-1α), interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α) (Calandra et al. 1990; Loppnow et al. 1990; Bendtzen, 1988). Picomolar concentrations of LPS have been shown to induce TNF-α production 30-60 minutes after intravenous injection of LPS (Beutler and Cerami, 1986).

It has been shown that certain serum proteins can enhance the cytokine stimulation ability of LPS (Obayashi et al. 1986; Warren et al. 1986). Tobias has reported the discovery of a 60KDa acute phase LPS binding serum glycoprotein synthesised by hepatocytes (Tobias et al. 1986). This LPS-binding protein (LBP) binds tightly to the lipid A region of LPS (Tobias et al. 1989). LBP has been shown to have a high amino acid similarity with bactericidal/permeability-increasing protein (BPI) (Gray et al. 1989). However, despite their similarities in amino acid sequence and their ability to bind to LPS, LBP and BPI have significantly different biological consequence (Ulevitch and Tobias, 1994). LBP greatly enhances the biological effects of LPS, by reducing the threshold for LPS's effect by 1000 fold, and the rate of cytokine production is also markedly accelerated (Ulevitch, 1993). In contrast BPI inhibits the ability of LPS to stimulate cells. Furthermore, when in contact with intact bacteria, BPI has a bactericidal activity, whereas LBP acts as an opsonin.

A number of researchers have reported the presence of an 80KDa LPS binding protein on the plasma membranes of a variety of endotoxin responsive cells (Lei and Morrison, 1988). Tobias *et al* have provided more conclusive evidence for the binding of a LPS-LBP complex to a 50-55KDa glycosylphosphatidyl inositol (GPI)-anchored membrane glycoprotein expressed on myeloid cells (membrane CD14; mCD14) (Wright et al. 1990) and to a soluble form lacking the GPI anchor (soluble CD14; sCD14)(Bazii et al. 1986). Lee *et al* have clearly shown the importance of CD14 in controlling LPS responses and have provided evidence that LPS exerts its effects via CD14-dependent pathways (Lee et al. 1992). The same group has also provided evidence suggesting that binding of endotoxin to cell surface CD14 is followed by subsequent interactions with additional membrane protein(s) that initiate trans-membrane signalling (Lee et al. 1993). It is of interest that mCD14, a GPI-anchored protein that does not directly communicate with the cell interior, can mediate trans-membrane signalling.

The inhibition of LPS by Nephronin in the LAL assay is thought to be due to the chemical modification of the lipid A moiety by Nephronin. This interaction may in turn prevent the binding of LPS to LBP, thereby inhibiting cytokine production by MØ and other LPS responsive cells.

SUMMARY OF THE INVENTION

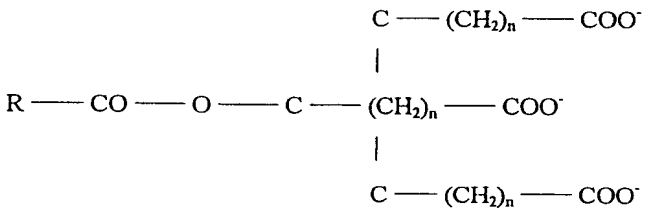
In a first aspect the present invention consists in a non-proteinaceous compound isolatable from the urine of patients suffering from steroid responsive nephrotic syndrome, the compound having the following characterising features:

(i) a molecular weight less than 1 KDa;

(ii) binds specifically to heparin and heparan sulphate but not other glycosaminoglycans; and

(iii) inhibits LPS induced production of TNF-α and/or IL-1α.

In a second aspect the present invention consists in a compound having the general formula:



in which R is a carbon chain of up to 22 carbon atoms which be saturated or unsaturated, branched or cyclic, n may be the same or different and is an integer of 0 to 10
and pharmaceutically acceptable salts and derivatives thereof.

In a preferred embodiment of the present invention R is CH_3 or $CH_3(CH_2)_n$ where n is 1 to 18, preferably 1 to 8.

In a third aspect the present invention consists in a composition comprising the compound of the first or second aspect of the present invention and a pharmaceutically acceptable carrier.

In a fourth aspect the present invention consists in a method of treating, preventing, or reducing the risk of Gram negative septic shock or another disease state involving elevated cytokine levels in a subject comprising administering to the subject an effective amount of the composition of the third aspect of the present invention.

In a fifth aspect the present invention consists in a method of treating, preventing, or reducing the risk of viral in a subject comprising administering to the subject an effective amount of the composition of the third aspect of the present invention.

In a preferred embodiment of the fifth aspect of the present invention the virus is Herpes virus or HIV.

DETAILED DESCRIPTION

In order that the nature of the present invention may be more clearly understood preferred forms thereof will now be described with reference to the following non-limiting Examples.

Materials and Methods.

Chemicals

All chemicals were purchased from Sigma Aldrich, unless otherwise stated.

Purification of the factor

Urine, from SRNS patients in relapse, was collected in untreated clean 24-hour collection bottles. The urine bottles were then stored at -20°C until used. The protein content of urine samples was determined using Multistix SG (Bayer Diagnostic UK LTD, Basingstoke, Hampshire, UK). Urine samples with protein content of 3+ were used for the purification of the factor.

Up to 2 litres of urine was thawed, concentrated and dialysed extensively against milli-Q water using an Amicon spiral cartridge concentrator/filtration system with a cut-off of 10,000 dalton. The concentrated urine was then freeze-dried and the lyophilised powder resuspended in 0.1M ammonium acetate buffer pH 9.0. The pH of the sample was then adjusted to pH 9.0 with a solution of 10% ammonium hydroxide. The resuspended sample was centrifuged at 30,000g for 20min. at 4°C, prior to column chromatography.

The clear supernatant was loaded onto a 300 ml Fast-Q Sepharose column (Pharmacia, St Albans, Herts, UK) previously pre-equilibrated with 0.1M ammonium acetate buffer pH 9.0 at 4°C.

The column was run isocratically at 4°C. Material eluted unbound to the column under these conditions were pooled and freeze-dried. The bound material was eluted from the column with 2M NaCl in the same buffer and discarded.

The freeze-dried material was then resuspended in 20-50 ml of 0.1M sodium citrate pH 3.5. The pH of the sample was adjusted to pH 3.5 with 1M citric acid. The resultant cloudy solution was centrifuged at 30,000g for 20min at 4°C and the clear supernatant was then loaded onto a Hiload 26/10 S-Sepharose high performance

column, previously pre-equilibrated with 0.1M sodium citrate pH 3.5. The sample was loaded onto the column at 2ml/min, followed by isocratic elution of unbound material by 0.1M sodium citrate until the base line was achieved. The bound material was eluted with step elution of 10, 25 and 100% of the same buffer containing 2M NaCl and 5 ml fractions were collected. Fractions eluted with the 25% salt step (0.5 M NaCl in 0.1 sodium citrate) were pooled and dialysed extensively against milli-Q water.

The pooled material was then freeze-dried and the lyophilised material was resuspended in 0.1M-ammonium acetate pH 9.0. The pH was adjusted to pH 9.0 with a solution of ammonium hydroxide and was then loaded onto a Q-Sepharose column. Under these conditions, the majority of the material bound to the column. After loading the column with the sample, the column was washed with at least 4-column volume of water. Finally the bound material was eluted as a single broad peak with 3.0% formic acid. This material was freeze-dried several times to remove the formic acid buffer. The lyophilised material was resuspended in water and pH adjusted to neutral with a 10% solution of ammonia.

This material appeared to be non-proteinaceous, since standard protein estimation assays failed to show the presence of any protein. This was further confirmed by the fact that this material did not absorb at 280nm. Spectral analysis showed maximum peak at approx 205-215nm.

Cellulose acetate electrophoresis

Flat-bed cellulose acetate electrophoresis was used for the detection of material that might bind to different glycosaminoglycans and inhibit their migration. Cellulose acetate (Sartorius Limited, Epsom, Surrey, UK.) sheets of 15cm by 7.5cm were pre-soaked in the running buffer, 0.1M Barium acetate pH 6.9. The excess buffer was removed from the acetate sheet by placing the sheet between two sheets of tissue paper. The sheet was then positioned in the electrophoresis tank and 0.1 units of glycosaminoglycans, in a volume of up to 1 µl, was loaded onto numbered positions no more than 2 mm in diameter on the cellulose acetate sheet and dried under the heat of a lamp.

To test the binding ability of the samples (factor) to GAGs, in particular heparin, generally up to 2µl of each sample was loaded on to the GAG spot and dried under the heat of a lamp. However, in cases where the samples were particularly dilute, up to 10µl of each sample was loaded onto each spot in 2µl aliquot. The spots were dried under a lamp prior to further loadings.

Cellulose acetate electrophoresis was performed at 60 volts for 3 hours towards anode.

The GAGs on the cellulose acetate sheet were then visualised by staining with a solution of Alcian Blue 8GX (0.05% Alcian blue in 50mM sodium acetate pH 5.8 containing 50mM $MgCl_2$). The excess dye was washed from the cellulose acetate sheet with a solution of 5% acetic acid. The cross-reactivity of the GAGs with the samples was assessed by the inhibition in migration compared with the migration of untreated corresponding GAG.

Fig 2 shows the specific binding of Nephronin to Heparin and heparan sulphate but not chondroitin and dermatan sulphate. This is demonstrated by the inhibition in migration of these glycosaminoglycans. The inhibition in migration of heparin and heparan sulphate is ascribed to specific interaction of Nephronin with these molecules, rather than on the basis of charge, since all these molecules are highly negatively charged.

Endothelial cells.

Endothelial cells were obtained from human umbilical veins by collagenase (0.1%) digestion as previously described (Jaffe et al. 1973) and modified by Klein et al (Klein et al. 1993).

Histochemical studies

Endothelial cells were grown to confluence on gelatinised cover slips. Once confluence was achieved the cover slips were removed and the cells were washed with phosphate buffered saline (PBS) at 37°C. The cells were then fixed with 4% paraformaldehyde in PBS solution. For specific staining of heparan sulphate, the staining procedure was performed at pH 1.9. Under these conditions all proteinaceous material is positively charged, whilst heparan sulphate moieties remain negatively charged. 5nm gold-conjugated poly-L-lysine probe particles (Biocell Research Laboratories, Cardiff, UK) were used for specific staining of heparan sulphate, based on charge interaction, essentially as described by the manufacturers. The cells were counter stained in Meyer's Haematoxylin for 1min prior to mounting in Aquamount (BDH, UK).

Coagulation studies

Extrinsic, UPTT and intrinsic, KPTT were performed as directed by the suppliers (Diagnostic Reagents Limited, Thames, Oxon, UK). Commercially available Kits for the determination of heparin like molecules based on the antithrombin III activity for the inhibition of thrombin formation were used for the measurement of the factor, as directed by the suppliers (KABI Diagnostica, Supplied by Quadratech, Surrey, UK).

SDS-PAGE

Polypeptides were separated by SDS-PAGE [Laemmli, 1971 #72], electroblotted on to nitrocellulose sheets (Towbin et al. 1979). The blotted protein bands were visualised on the nitrocellulose sheet with amido black staining solution [0.2% (w/v) amido black, 25% (v/v) isopropanol, 10% acetic acid], and de-stained in the same solution without dye.

The protein bands were sent to Ludwig Institute for Cancer research, Riding house street, London, UK, for end terminal amino acid sequencing.

Thin layer chromatography and staining for peptides, lipids, carbohydrates and oligosaccharides were performed as previously described (Dawson et al. 1986).

Protein estimation was carried out using the Bradford method (Bradford, 1976).

Characterisation of Nephronin

Purification of the hypothesised circulatory highly cationic protein clearly showed that this protein was of immunoglobulin origin (Fig 1), and that its existence was an artefact. However, there is a considerable amount of evidence to suggest that the proteinuria in nephrotic syndrome is due to a reduction in the negative charge of heparan sulphate on the glomerular capillary wall, brought about by a circulatory factor. During the search for the cationic protein, a number of assay methods for the detection of heparin binding molecules were developed.

A method for detecting Nephronin, was to use a one dimensional cellulose acetate electrophoresis, that is often used in the characterisation of GAGs (Klein et al. 1992). However, the data obtained using cellulose acetate electrophoresis must be treated cautiously, since false positive data can be obtained with high concentrations of salts, including sodium citrate.

Using the modified Cellulose acetate electrophoresis for the detection of a heparin binding factor, a factor was isolated from the urine of children with SRNS, that binds to and inhibits the migration of glycosaminoglycan, heparin. Further studies for the specificity of this factor for binding other GAGs, clearly showed that this factor only inhibited the migration of heparin and heparan sulphate but not other glycosaminoglycans on cellulose acetate electrophoresis (Fig 2).

Nephronin was found to be highly negatively charged based on its tight binding to anion exchange columns (Q-column) at neutral pH. As well as the cellulose acetate electrophoresis for its detection, thin layer chromatography was performed for the identification of various components associated with Nephronin during the purification procedure. Staining of this factor for peptides, carbohydrates and lipids at various stages of purification clearly demonstrated that Nephronin was tightly bound to lipids. The lipid contamination proved to be a great obstacle in the purification of Nephronin, and for a long period the Heparin/heparan sulphate binding ability of Nephronin was ascribed to the lipid entity.

Standard chloroform/methanol extraction for the separation of the lipid entity, resulted in a huge reduction in the activity of the factor, with the active material in the aqueous layer free from the lipid entity.

Nephronin was then further purified by a number of other chromatographic techniques and the active fractions retained on the basis of their binding to heparin and inhibition of its migration on cellulose acetate electrophoresis.

During the purification of Nephronin, amicon stirred cell ultrafiltration with membranes of 30, 10 and 1kDa cut off were used. These studies indicated that Nephronin had a relatively large molecular weight. However, under reducing conditions, Nephronin was found to pass through membranes with a cut of 1kDa. Gel filtration studies under reducing conditions also indicated a molecular size of less than 2KDa.

Thin layer chromatography and staining for carbohydrates, lipids and oligosaccharides, as well as phenol-sulphuric acid assay (Dubois et al. 1956) for carbohydrate determination were negative. Pronase digestion of the factor also has no effect on its heparin binding, inhibition of its migration on cellulose acetate electrophoresis.

Cross reactivity of Nephronin with heparan sulphate on the endothelial cells

Although the specificity of Nephronin for heparin and heparan sulphate was shown using the cellulose acetate electrophoresis method, it was important to establish the effects of this material on endothelial cells, since the GAG coating the endothelial cells has been shown to be almost exclusively heparan sulphate (Kanwar, 1984). The binding of Nephronin to heparan sulphate on endothelial cells was studied on paraformaldehyde pre-fixed cells and then viable cells (Fig 3). Plate A shows staining of heparan sulphate on the surface of endothelial cells. Plate B shows the same staining on the surface of endothelial cells after treatment with Nephronin.

1. **Effect of the factor on fixed cells.**

Exposure of paraformaldehyde fixed endothelial cells to isolated Nephronin, diluted in PBS, showed a considerable reduction in the number of anionic sites. Fig 3 clearly demonstrates that the treatment of endothelial cells with Nephronin abolishes the characteristic staining for heparan sulphate on the untreated endothelial cells.

2. **Effect of the factor on viable cells.**

In order to test the effect of the factor on cell surface heparan sulphate of viable endothelial cells, the factor was added to the culture medium (up to 5% v/v). Initial experiments showed that exposure of the viable cells to the factor caused the dissociation of the cells from the substratum. To minimise the dissociation of the cells from the substratum, the incubation period was reduced from 1 hour to 10min. The cells were then stained for surface anionic sites as above.

3. **Effect of the factor on the viability of the endothelial cells.**

To assess the binding of Nephronin to heparan sulphate on viable endothelial cells, Nephronin was added to confluent endothelial cells. In the initial experiments the endothelial cells were exposed to Nephronin for one hour as described above. However, it was found that during this period almost all of the endothelial cells were detached from the culture plate. To investigate whether Nephronin had caused cell death, resulting in the separation of cells from the substratum, the culture medium containing the cells was removed and centrifuged

at 1000rpm for 10min. The pelleted cells were resuspended in cold PBS and washed twice with cold PBS. Trypan blue staining of the cells showed that over 95% of the cells were viable.

To continue the investigation for the binding of Nephronin with the cell surface heparan sulphate, the incubation time was reduced to 15 minutes and the amount of Nephronin added was reduced to 2.5% of the culture volume. Fig 4 A shows the GAG staining on untreated cells under these conditions. Fig 4B clearly shows a considerable reduction in the GAG staining of the cells treated with the factor.

Cross-reactivity of Nephronin with heparin in coagulation studies.

Having established Nephronin's ability to bind to heparin and heparan sulphate, it was important to establish whether this material had any effect on blood coagulation.

Both extrinsic, UPTT and intrinsic, KPTT coagulation systems were used. Nephronin was found to prolong coagulation time in much the same way as heparin. Furthermore, the anti-coagulant effect of Nephronin was found to be synergistic with heparin.

To further investigate this relationship with heparin, a commercially available chromogenic kit for the measurement of heparin like molecules, based on the acceleration of antithrombin III activity for the inhibition of thrombin formation was used (Griffith, 1986; Bjork et al. 1986). Once again, it was found that Nephronin behaved in much the same way as heparin and in synergy with heparin. These findings suggest that Nephronin may be able to self-polymerise, since activation of antithrombin III requires at least a pentasaccharide sequence of heparin (Jackson et al. 1991).

Identification of the cells involved in the production (secretion) of Nephronin

Although the pathogenesis of SRNS is still unknown, it is well known that patients with SRNS go into relapse with upper respiratory and minor infections. As mentioned earlier the loss in immune regulation is thought to play an important part in the pathogenesis of the disease.

To test whether the immune cells from SRSN patients during relapse produce (secret) Nephronin without mitogen stimulation of the cells, and whether it is possible to induce immune cells from normal subjects to produce Nephronin, PBMC from SRNS patients and normal subjects were isolated.

Methods

Isolation of PBMC

Whole blood (10 ml) from SRNS patients and healthy volunteers was collected into 50 ml sterile Greiner tubes (Greiner Laboratory, Dursley, Glos. UK) containing 1ml sterile sodium citrate / citric acid (3 parts 0.1M sodium citrate, 2 parts 0.1M citric acid) anticoagulant and diluted 1:3 with sterile PBS. The 33.0 ml

PBS/blood/anticoagulant was layered onto 16.5 ml Ficoll/hypaque, Pharmacia, St Albans, herts, UK) and centrifuged at 1800g for 30min at 4°C. The cell layer at the interface between ficoll and plasma was removed, diluted 1:5 v/v with ice cold PBS and centrifuged at 750g for 10min at 4°C. The pelleted cells were washed once more with cold PBS and the final cell pellet was resuspended in 5 ml of RPMI 1640 culture medium (Gibco, Paisley, Scotland, UK) containing 10% heat inactivated foetal calf serum (FCS) (Sigma chemical company, Poole, Dorset, UK). The cells were then aliquoted in 8-well culture plates (Nunc, Paisley, Scotland, UK) for 45min at 37°C in incubators supplied with air, 5% CO_2, to allow macrophages to adhere to the plastic. Following adherence, the cells suspension were removed and washed with pre-warmed RPMI 1640 at 37°C and maintained in the same culture medium supplemented with 50 units per ml of IL-2 (Zymed Laboratory INC., distributed by Cambridge Bioscience, Cambridge, UK.).

Production of Nephronin by human lymphocytes.

PBMC isolated from SRNS patients were maintained in RPMI 1640 culture medium containing 10% v/v heat inactivated FCS and 50units/ml of IL-2. The culture medium was removed at 24, 48 or 72 hour time periods and stored frozen at -70°C until use.

The culture mediums were then processed analytically for the purification of Nephronin.

To assess the effect of the Nephronin on unstimulated PBMC, PBMC were also isolated from healthy volunteers. The PBMC were maintained as above except that on these occasions the culture media were supplemented with 10% SRNS patient's plasma. For control studies PBMC isolated from healthy subjects were cultured as above and supplemented with 10% normal plasma.

Plasma from SRNS and healthy subjects were prepared by mixing 10 ml of whole blood with 1ml of sterile sodium citrate / citric acid (3 parts 0.1M sodium citrate, 2 parts 0.1M citric acid) as anticoagulant. The cells were removed by centrifugation at 850g for 10min. The clear plasma was removed and stored in small aliquots at -70°C. The pelleted cells were usually used for other related experiments.

Results

The data presented in Table 3 clearly demonstrates that, lymphocytes isolated from SRNS patients during relapse are capable of producing Nephronin. Furthermore, addition of patients plasma, but not plasma from normal subjects, to cultures of lymphocytes from normal subjects also induced these cells to produce Nephronin. These results suggests

1. Lymphocytes from SRNS patients during relapse produce Nephronin
2. There is a circulatory factor in the plasma of these patients that can stimulate lymphocytes isolated from normal subjects, to produce Nephronin.

The fact that the immune cells from SRNS patient during relapse produce Nephronin, indicates the importance of this compound as part of the immune response to infection. However, the lack of immune regulation, as suggested by many workers in the field, may be responsible for the on set of nephrotic syndrome.

Isolation of Nephronin from urine of meningitis patients.

The studies above indicated that the immune cells in response to infection could produce Nephronin. To test this hypothesis, urine from three children with meningitis and two normal children were processed for analytical isolation of Nephronin. Nephronin was isolated from urine of two of the meningitis patient who had survived the disease. The urine of the patient that did not contain Nephronin did not survive the disease.

Nephronin was not isolated from the urine of normal children.

Endotoxin neutralising ability of Nephronin.

Because of the anionic nature of Nephronin and its ability to bind to lipids, it was postulated that this factor might behave as a cationic detergent. Furthermore, the fact that Nephronin was also isolated from urine of children with meningitis suggested that this factor is produced in response to infection. Consequently, Nephronin was thought to behave similarly to that of the cationic antibiotic, polymyxin.

Limulus Amoebocyte Lysate (LAL), utilises the E.coli endotoxin for the activation of a pro-enzyme which then hydrolyses a substrate to yield a chromogenic product. Fig 5 shows the principle of LAL activation. This system was used to demonstrate the possible anti-endotoxin ability of Nephronin.

Table 1 show a representative set of data for the inhibition of endotoxin by Nephronin.

Agglutination of gram-negative bacteria.

Given the fact that Nephronin inhibited LPS by the LAL assay, indicated that this compound might bind to LPS molecule and neutralise it effect by direct physical binding. To test whether, binding of Nephronin to LPS had an effect on bacterial agglutination, a number of gram negative and gram positive bacteria were used to test this hypothesis. Furthermore, since LPS molecule is not always exposed in different strains of gram negative bacteria, the agglutination of Nephronin on heat and/or penicillin killed bacteria was also test. Table 2 shows the agglutination of gram negative but not gram positive bacteria by Nephronin. It is noteworthy that in the cases of heat and penicillin killed bacteria, agglutination occurred very quickly, whereas where viable bacteria were used, this was not always readily apparent.

Physicochemical Characterisation of Nephronin

Preliminary characterisation of Nephronin suggested the absence of peptide bonds involved in its heparin binding ability, since this activity is not affected by treatment with 6M HCl and heating at 105°C for 24h. Furthermore, thin layer chromatography and staining for carbohydrates, lipids and oligosaccharide, as well as phenol-sulphuric acid assay for carbohydrate determination were negative. Pronase digestion of Nephronin also seems to have no effect on its heparin binding and inhibition of its migration on cellulose acetate electrophoresis.

Elemental analysis tests for Carbon, Hydrogen, Phosphorous and sulphur of Nephronin, only showed the presence of 37.04% carbon and 3.74% hydrogen.

NMR and mass spectroscopy of the isolated Nephronin were inconclusive. However, the presence of citrate was clearly demonstrated on NMR. FAB analysis suggested the presence of two and three carbon molecules.

These initial structural studies suggested that Nephronin might be easily hydrolysed. However, based on the information available, a plausible structure for Nephronin was hypothesised. Methodology for synthesising this compound was then proposed, and recently, successful synthesis of Nephronin has been achieved. Attempts at the synthesis of Nephronin are described as experiments.

Establishment of a biological system

To test the effect of Nephronin in a biological system, the products from different synthesis attempts, were tested on human lymphocytes, isolated from blood buffy coat (concentrated White Blood Cell preparation, supplied by the Blood Bank). Lymphocytes were stimulated with endotoxin (*E.coli*, serotype 055:B5, Sigma-Aldrich chemical company) in the presence and absence of the synthesised compounds. For positive controls, a number of other biological stimulators were also used. These included, PHA, Concanavalin A, streptococcal bacterial membrane protein (M protein) and enterotoxins (superantigens) SpeA and SpeB.

Sandwich ELISA for the measurement of mitogen induced cytokines was established. Sandwich ELISA for TNF-α, and IL-1α was routinely used for the measurement of these cytokines in the culture supernatant of the activated cells post-stimulation. Cells were harvested at 24, 48 and 72 hours post-stimulation and the culture supernatant's were used in the determination of the cytokine levels.

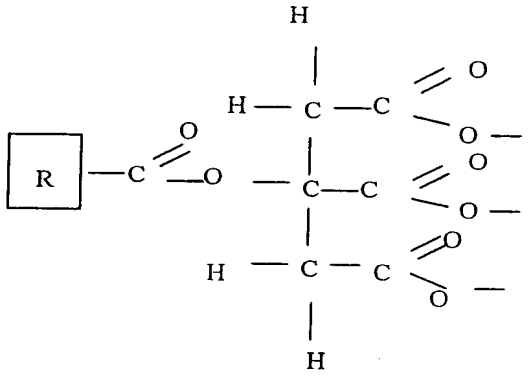
Specific antibodies were purchased from a number of suppliers; mouse monoclonal and rabbit polyclonal antibodies against human TNF-α were purchased from Genzyme, USA, supplied by Lab Supply, Australia. Mouse monoclonal and rabbit polyclonal antibodies against human IL-1α, IL-4, IL-6 and interferon-γ (IFN-γ) were purchased from Endogen, USA, supplied by CSL Biosciences. Horseradish peroxidase conjugated anti-rabbit antibodies were purchased from Zymed, USA.

Cytokines used in the standard curves were purchase from Peprotech INC, supplied by Australian Laboratory Services Ltd.

Synthesis of Nephronin

The hypothesised structure for Nephronin assumes that this compound is detergent like in structure with specificity towards certain sugar moieties, hence an anionic molecule that behaves like a cationic detergent. Taking this into consideration, one can then visualise Nephronin being able to inhibit LPS.

NMR studies on the isolated Nephronin had demonstrated the presence of citrate (citric acid). The structure hypothesised for Nephronin is based on the esterification of a fatty acid molecule on the hydroxyl group of the citrate molecule.



The structure above gives the hypothetical structure for Nephronin. The **-R** group represents the carbon chain length of the fatty acid.

Methods for synthesising Nephronin.

Initial attempts

Initial attempts at synthesising the hypothesised structure of Nephronin, were to simply mix the fatty acid and citric acid in an aqueous solution. Since long chain fatty acids were used in these initial attempts, solubility of the acids in water posed a problem. Palmitic acid was the first fatty acids used in these procedures. There are three –COOH groups on the citric acid molecule, therefore a ratio of 3:1 fatty acid to citric acid was used. For example, to a tube containing 30m of citric acid dissolved in 2ml of water, 100mg of palmitic acid was added. To ensure mixing of the fatty acid with the citric acid, the reaction tube was heated to 80°C so as to melt the

fatty acid. However, once the reaction tube was cooled to room temperature, the fatty acid precipitated out of the solution. Interestingly, it was found that, heating the mixture of fatty acids in a solution of 0.2M citric acid at 80°C for 48h, resulted in the formation of a clear solution at room temperature. This is thought to be due to the formation of small fatty acid vesicles. Similar observations were also made for, Oleic acid, pentadecanoic acid, but not for Linoleic acid. After this period, the pH of the samples was adjusted to neutral with 2M NaOH. Testing of these samples did not show any inhibition of LPS-induced cytokine production.

Use of Phosphorus Oxychloride.

To synthesise the hypothesised Nephronin, it was thought necessary to activate the fatty acid to acid chloride, so that the activated fatty acid could form an ester with the citrate molecule.

Phosphorus oxychloride (POCl3) was chosen to convert fatty acids to acid chloride (R-C=O-Cl). The acid chloride when added to the citrate should then form an ester with the –OH group on carbon 3.

Phosphorus oxychloride reacts violently with water to form hydrochloric acid (HCl). To prevent this reaction occurring, the entire procedure was performed in the absence of water.

Initial Attempts at synthesising Nephronin

Long chain fatty acids were chosen to synthesise Nephronin, these included the following,

1. Palmitic acid
2. Oleic acid
3. Linoleic acid
4. Pentadecanoic acid

Initially, POCl3 was added directly to the fatty acid and mixed. Since there are three –Cl groups on the POCl3, a ratio of 3:1 of fatty acid to POCl3 was used. Taking into consideration the molar/molar concentration of the fatty acid and the POCl3, the volume of POCl3 was not enough to allow thorough mixing of the mixture. To overcome this problem an excess of POCl3 was added to the fatty acids.

To facilitate the synthesis, the tubes containing the mixture were heated to 80°C for approx 30minutes so that the fatty acids melted. The mixture changed into a colourless liquid, however, after standing at room temperature, the whole reaction mixture became one solid mass.

An alternative approach was to melt the fatty acid by heating to 80°C prior to the addition of POCl3. The mixture was then incubated at 80°C over night with constant mixing. The colourless mixture had changed colour to a deep red solution. 33mg of Na3Citrate dissolved in 50µl of water was added to this solution, but the two liquids did not mix. The change in colour is thought to be due oxidation of some of the components in the reaction tube, most likely POCl3. Once again it was not possible to test these samples.

It was decided to perform the reaction in chloroform as the organic phase. Although the fatty acids dissolved readily in chloroform, Na3Citrate did not dissolve and remained as crystals. It was assumed that for a reaction to proceed, it was necessary for all reactants to be in a liquid phase. Trial of solubility of the reactants showed that citric acid, but not Na3Citrate, dissolved in acetonitrile when heated to 80°C for 10-20min. However, palmitic acid was not very soluble in acetonitrile. Consequently, Fatty acids dissolved in chloroform were mixed with citric acid dissolved in acetonitrile.

A range of combinations where the ratio of fatty acid dissolved in chloroform and citric acid dissolved in acetonitrile was tested. In each case the main problem was thought to be due to oxidation of some of the components of the reaction mixture, since in all cases the reaction mixture changed colour to red and very deep red. Furthermore, the resulting compounds did not dissolve in water. Because of solubility problems, these preparations were not tested for their biological activity.

Acetonitrile alone as the solvent of choice was also used to synthesise Nephronin, but with no effect. Given the fact that Na3Citrate is not soluble in acetonitrile, citric acid was chosen.

For example, 100mg palmitic acid dissolved in 4ml of acetonitrile. To this 100µl of POCl3 was added and after 30min incubation at room temperature with mixing, 40mg of citric acid was added. The reaction was allowed to proceed at room temperature (RT). After this time the samples were freeze-dried. The lyophilised material was found to be very insoluble in water, even after adjusting the pH of the samples to neutral. This was thought to be due to the excess amount of fatty acid present in the reaction mixture. Consequently, the presence of water insoluble fatty acid might interfere with Nephronin, since Nephronin molecules are likely to bind tightly with the fatty acid.

A variation of this reaction procedure, based on the idea that upon formation of the synthesised compound, it may be possible to remove the product from the reaction mixture, leaving the fatty acid in the solvent.

In this approach, the reaction was performed essentially as above. After over night incubation at RT, excess volume of acetonitrile was add to the reaction tubes. In an attempt to specifically precipitate the synthesised compound, and neutralise the acid, excess solid NaHCO3 was added to the mixtures. After regular mixing of the tubes, the mixtures became cloudy within 1h. The reaction was allowed to proceed with mixing for several hours. The reaction tubes were centrifuged at 4000 rpm for 10min and the resulting pellets were allowed to air dry in a fume hood. The pellets were dissolved in water and the pH of samples adjusted to neutral with 0.1M citric acid. Testing of these samples did not show any biological activity.

The solvent from each reaction tube was also dried. The lyophilised material was found not to dissolve in water. Consequently, these samples were not tested.

A considerable amount of time and effort was put into synthesising the compound using essentially the methodology described above. However, none of the preparations were effective in neutralising LPS induced cytokine production.

Conclusions from the early synthesis attempts

1. The fact that the solvents containing the POCl3 reaction mixture changed colour to red suggested that some components of the reaction were being oxidised, most likely POCl3. Consequently, it was decided to perform the reaction under nitrogen.

To test which particular solvent was most appropriate with the least amount of oxidation, a number of solvents were mixed with POCl3 and mixed in air. The rate of oxidation of POCl3 was taken to be proportional to the time taken for colour development.

The solvents tested

Chloroform
Acetonitrile
Acetone
Isopentane
Hexane

Maximum colour change was observed for chloroform, where a deep red colour was observed very quickly. However, addition of POCl3 to hexane resulted in minimal amount of colour change with time. After 24h incubation at RT, only a slight tan colour was observed for hexane-POCl3 mixture.

2. Since the pH of the mixture was very acidic, to reduce the acid effect, Na3Citrate was used in the synthesis procedure.

The experiment numbers refer to the order in which the experiments were carried out in the synthesis of Nephronin. The brief description given above summarises the attempts in experiments 1-8.

Experiment 9

The information gained from previous attempts was utilised in this experiment. Hexane was used as the solvent of choice and the tubes were capped under nitrogen.

The following reaction mixtures were set up. The fatty acids were first dissolved in the solvent prior to the addition of POCl3.

1. 100mg Palmitic acid in 5ml Hexane + 100µl POCl3
2. 10mg Oleic acid in 5ml Hexane + 100µl POCl3
3. 10mg Pentadecanoic acid in 5ml Hexane + 100µl POCl3
4. 10mg Linoleic acid in 5ml Hexane + 100µl POCl3

5. Control, No fatty acid was used. 5ml Hexane + 100µl POCl3

The tubes were capped under nitrogen and placed on a roller to allow mixing of the samples. After 30min incubation at RT, 0.4g of Na3Citrate was added to each tube. Once again the tubes were capped under nitrogen and placed on a roller for mixing. After 24h incubation at RT, the content of the tubes were freeze-dried. To each tube 2ml of Hexane was added and the content of each tube was mixed thoroughly and hexane was evaporated under nitrogen. 3ml of water was added to the lyophilised samples. The samples did not dissolve in water. These samples were freeze-dried and 10ml of water was added to the lyophilised material. To ensure full solubility of the samples, the tubes were heated to 80°C for 30min with mixing on a roller. The samples had an average of pH 6.3. The pH of these samples was adjusted to neutral with 0.2M Na3Citrate. The fine adjustments to pH 7.4 were made with 2M NaOH. A considerable amount of the lipids remained as a layer on top of the solution. To test these samples for their biological activity, aliquot of each preparation was autoclaved.

Results

Fig 1 shows the effect of different preparations of Nephronin using long chain fatty acids. Although the synthesis procedure was identical in all cases, the only preparation that inhibited LPS-induced TNF-α production was that of pentadecanoic acid. This preparation inhibited TNF-α by over 80% 24h post-stimulation. Similar results were obtained for IL-1α, where pentadecanoic preparation of Nephronin inhibited LPS-induced IL-1α production by over 50%, 24h post-stimulation. It is noteworthy that the levels of IL-1α produced for other preparations were higher than cells stimulated with LPS alone.
The inhibition of TNF-α and IL-1α by pentadecanoic acid preparation, were considerably reduced by 72h post-stimulation.

Conclusions

The results from experiment 9 showed that it was possible to synthesis biologically active Nephronin. However, the fact that fatty acids with longer carbon chain used in the synthesis of Nephronin were not able to inhibit LPS-induced cytokine production, suggests that these longer chain fatty acids were unable to form the ester. It may be necessary to optimise the synthesis procedure for these fatty acids at a later date.

Experiment 10

Aims

1. To test whether using tri-sodium citrate (Na3Citrate) or citric acid would make a difference in synthesising a biologically active product.

2. To test whether increasing the concentration of POCl3 increased the efficiency of the synthesis.

The following reaction mixtures were set up.

In each reaction, the fatty acid was first dissolved in hexane, followed by the addition of POCl3. The tubes were capped under nitrogen and after 1-hour incubation at room temperature, the reaction mixtures were then added to appropriately numbered tubes containing either tri-sodium citrate (Na3Citrate) or citric acid.

1. 30mg Pentadecanoic acid dissolved in 2ml hexane then 30µl of POCl3 + 0.2g NaCitrate.
2. 30mg Pentadecanoic acid dissolved in 2ml hexane then 30µl of POCl3 +0.15g Citric acid.
3. 30mg Pentadecanoic acid dissolved in 2ml hexane then 60µl of POCl3 +0.2g Na3Citrate.
4. 30mg Pentadecanoic acid dissolved in 2ml hexane then 60µl of POCl3 +0.15g Citric acid.

The reason that 0.15g of citric acid was used compared to 0.2g of Na Citrate, was to account for the weight of sodium.

Once again the tubes were capped under nitrogen and the reaction allowed to proceed at room temperature whilst being mixed on a rotator for 2 hours. All samples were then freeze-dried. 2ml of hexane was added to the lyophilised material to remove excess POCl3. The hexane was then evaporated under vacuum. Finally the samples were resuspended in 1ml of water and freeze-dried.

The lyophilised material was resuspended in 2ml of water and pH adjusted to pH 7.0 (neutral) with NaOH. Where Na3Citrate was used, a considerable amount of material did not dissolve in water, however, where citric acid was used, the material remained stocked to the walls of the tubes whilst the solution remained clear. In the case of Na3Citrate, all of the material seemed to resuspend in water, but not actually dissolving.

Taking advantage of previous observations, where heating the fatty acid mixture to 80°C for a period longer than 24h resulted in the clarification of fatty acid in water, the samples were heated at 80°C with mixing on a roller for 42h. After this period, a clear solution was visible in the tubes. However, it is noteworthy that the otherwise clear polypropylene tubes had become opaque. There are two possibilities for this, the first may be due to the effect of temperature over a prolonged period on the tubes. An alternative explanation is that excess fatty acid may have precipitated on the walls of the tubes.

These samples were autoclaved prior to being tested for their biological activity.

Results

The material synthesised (produced) under the conditions described above was ineffective in inhibiting LPS-induced TNF-α and IL-1α production. However, these same samples were very effective in inhibiting ConA and PHA –induced TNF-α and IL-1α production. At this stage, this property may be ascribed to the ability of citrate to inhibit these mitogens rather than the material synthesised this way.

Experiment 11

This experiment is essentially repeating experiment 10 with minor modifications.
The following reactions were set up.

1. 100mg pentadecanoic acid + 3ml hexane +100µl POCl3 + 0.4g Na3Citrate.
2. 100mg pentadecanoic acid + 3ml hexane +100µl POCl3 + 0.3g Na3Citrate.
3. 100mg pentadecanoic acid + 3ml hexane +200µl POCl3 + 0.4g Na3Citrate.
4. 100mg pentadecanoic acid + 3ml hexane +200µl POCl3 + 0.3g Na3Citrate.

Once the pentadecanoic acid was fully dissolved in the hexane, POCl3 was added to the reaction tubes. The tubes were capped under nitrogen and incubated at RT with mixing on roller for 1h, before the appropriate amount of citric acid or Na3Citrate was added to each tube and incubated at RT for 24h.

For controls the following reaction tubes were set up.

5. 100mg pentadecanoic acid +3ml hexane, No POCl3 was added + 0.4g NaCitrate.
6. 100mg pentadecanoic acid + 3ml hexane, No POCl3 was added +0.3g Citric acid.

On this occasion, when the pentadecanoic acid was dissolved in Hexane, the appropriate amount of citric acid or citrate was added to each tube. The tubes were capped under nitrogen and incubated with mixing on roller for 24h.

The samples were freeze-dried, by freezing the samples whilst still capped. To the lyophilised material 2ml of hexane was added, mixed vigorously and the hexane was evaporated under vacuum. The lyophilised material was dissolved in 1ml of milli-Q water, and freeze-dried. Finally the lyophilised material was resuspended in 3ml of PBS and the pH adjusted to pH 7.0 with NaOH. It was found that when these preparations were filter sterilised, the membrane retained a considerable amount of the material. This was due to the fact that these preparations did not dissolve in water to give a clear solution. Consequently, by filter sterilising these samples, it was very likely to remove the active portion of the products. Furthermore, it was thought that if these preparations were autoclaved, it would not lead to break down of the esters.

The samples were autoclaved prior to testing them for their biological activity. In each case 50µl of each sample was added to $1.5x10^6$ cell/ml of PBMC.

However, since the preparations formed precipitates in solution at RT, it was decided to pre-heat the samples to 50°C to melt the preparations, prior to addition to the culture plates. It is noteworthy that after addition of these samples to media at 37°C, a fine film could be seen on the surface of the culture media.

Results

All of the samples including the controls showed 100% inhibition of LPS, PHA and ConA induced TNF-α and IL-1α. Consequently, it was thought that this inhibition might be due to non-specific inhibition, since these preparations are likely to form vesicles engulfing the mitogens and preventing them from exerting their effects rather than specific inhibition of any one mitogen.

Experiment 12 (part 1)

At this time, I had also received Lauric acid (C12) and Myristic acid (C14). Since the results from previous experiments did not indicate which particular method may be more effective in synthesising Nephronin, both citric acid and Na3Citrate were used in the following synthesis. This experiment is essentially the repeat of the previous experiment.

1. 100mg Lauric acid + 3ml Hexane + 100µl POCl3 + 0.4g Na3Citrate.
2. 100mg Lauric acid + 3ml Hexane + 100µl POCl3 + 0.3g Citric acid.
3. 100mg Lauric acid + 3ml Hexane + no POCl3 + 0.4g Na3Citrate.
4. 100mg Lauric acid + 3ml Hexane + no POCl3 + 0.3g Citric acid.
5. 100mg Myristic acid + 3ml Hexane + 100µl POCl3 + 0.4g Na3Citrate.
6. 100mg Myristic acid + 3ml Hexane + 100µl POCl3 + 0.3g Citric acid.
7. 100mg Myristic acid + 3ml Hexane + no POCl3 + 0.4g Na3Citrate.
8. 100mg Myristic acid + 3ml Hexane + no POCl3 + 0.3g Citric acid.

The synthesis was performed as in experiment 11. The resulting products were found to be water insoluble. Given the fact that in the previous experiment, it was found that heating of the preparation to melt the fatty acids resulted in non-specific inhibition of mitogens, these samples were not tested.

Experiment 12, part 2

Whilst performing the above synthesis and considering that the final products were water insoluble, it became apparent that using long chain fatty acids may not lead to the compound of interest. At this particular time, the only available short chain acid was acetic acid.

Aims

1. To test whether either Na3citrate or Citric acid could be used for the synthesis of an active compound.
2. To demonstrate the effects of NaOH or ammonium hydroxide used in adjusting the pH of the products to Neutral.

Methods

The following reactions were set up.

1. 300µl acetic acid (glacial) + 2ml hexane + 100µl POCl3 + 0.4g Na3Citrate.
2. 300µl acetic acid (glacial) +2ml Hexane + 100µl POCl3 + 0.3g Citric acid.
3. 300µl acetic acid (glacial) +2ml hexane + no POCl3 + 0.4g Na3Citrate.
4. 300µl acetic acid (glacial) +2ml hexane + no POCl3 + 0.3g Citric acid.

In each case the acetic acid was added to hexane prior to the addition of POCl3. The tubes were capped under nitrogen and incubated at RT for 30min. The appropriate amount of Na3citrate or citric acid were weighed and added to each tube. The reactions were allowed to proceed at RT for 24h. The samples were then freeze-dried. 2ml of Hexane were then added to the resulting material, mixed vigorously and the hexane evaporated under vacuum. The lyophilised material was resuspended in 4ml of water.

In each case the samples were divided into two. The pH of one portion was adjusted to pH 7.0 with NaOH, and the other portion with a 10% solution of ammonium hydroxide. The final volume of each sample was adjusted to 5 ml.

On this occasion because of the length of the carbon chain of the acetic acids, the samples were filter sterilised using 0.2µ membranes. In each case 50 µl of each preparation was added to separate wells of a 24 well tissue culture plate, LPS was added to PBMC at $1.5x10^6$/ml to a final concentration of 1ng/ml LPS. The cells were mixed with LPS, and 1ml of PBMC containing LPS was added to each well. The time taken to dispense the cells into the culture plates was approximately 2-5 minutes.

The cells were harvested 24h post-stimulation by pipetting the culture media into 1.5ml eppendorf tubes followed by centrifugation at 2000rpm for 10 minutes. The supernantants were decanted into clearly marked tubes. The concentrations of TNF-α and IL-1α in the culture supernantants were determined by utilising sandwich ELISA.

To test whether the inhibition of the mitogen induced cytokine production was specific, the effect of these preparations on inhibition of streptococcal toxins and membrane protein (M-protein) was also tested. However, at this stage there was no information available on the effect of NaOH on these preparations. Consequently, the only samples that were tested were those that were pH adjusted to neutral with NaOH.

Results.

Figs 1 and 2 show the concentration of TNF-α and IL-1α of the culture supernatants 24h post-stimulation respectively. Preparations of Nephronin where Na3Citrate was used in the synthesis procedure and the pH of the final product adjusted to neutral with 10% ammonium hydroxide showed 66% inhibition of LPS-induced TNF-α and 100% of IL-1α production, 24h post-stimulation. For control, similar reactions were set up except that POCl3 was not used. The control preparations showed only 18% inhibition of LPS-induced TNF-α

production. In the case of IL-1α, an increase in the level of this cytokine was observed compared to LPS stimulation alone.

Interestingly, in preparations where similarly activated acetic acid was added to Citric acid, and the pH of the final product adjusted to neutral with 10% ammonium hydroxide, 49% inhibition of LPS-induced TNF-α production was observed compared to 45% inhibition for the control preparation. These preparations were not effective in inhibiting LPS-induced IL-1α production, and, in the case of the control preparation, it increased the IL-1α production by 63%. Similar results were obtained from the PBMC 48h post-stimulation, except that the inhibition of IL-1α had decreased to 76%.

In preparations where the pH of the samples was adjusted to neutral with NaOH, there was no significant detectable inhibition of the cytokine production. In fact in many cases, they were co-stimulatory for the production of IL-1α (results not shown).

Testing of preparations of Nephronin that were pH adjusted to neutral with NaOH, for inhibition of cytokine production stimulated by streptococcal membrane protein and toxins, showed that these samples were ineffective in inhibiting stimulation of PBMC by these mitogens (results not shown).

Effect of Nephronin on PHA-induced cytokine production.

50µl of different preparations of Nephronin was added to PBMC activated with 5µg/ml of PHA. The cells were harvested at 24 and 48h post-stimulation and the concentration of TNF-α and IL-1α were measured in the culture supernatants.

Figs 5 and 6 show the inhibition of PHA-induced cytokine production by preparations of Nephronin that were pH adjusted with 10% ammonium hydroxide solution. Preparation of Nephronin, where pre-activated acetic acid was added to Na3Citrate, showed 66 and 65% inhibition for TNF-α and 102 and 82% for IL-1α, at 24 and 48h respectively.

However, the preparation where citric acid was used in the synthesis of Nephronin, only 45 and 51% for TNF-α and 34% inhibition and 33% activation for IL-1α at 24 and 48h post-stimulation respectively.

Preparations of Nephronin where the pH was adjusted to neutral with NaOH showed some inhibition of the PHA-induced TNF- α production. It is noteworthy that the inhibitions observed were not significantly different from the control preparations. Interestingly, not only did these same preparations not show any inhibitory effect on IL-1α production however, they were stimulatory in nature.

Effect of Nephronin on streptococcal antigens

The preparations of Nephronin and controls, where the pH of the samples were adjusted to neutral with NaOH were tested for their effect on inhibition of streptococcal antigens. Streptococcal membrane protein, M-protein, and toxins, Spe A and Spe B were used because of availability. None of the preparations including the control preparations showed any inhibition of TNF-α and IL-1α. It is

noteworthy, that the preparations were added to the cell stimulated with the appropriate mitogens, the amount of TNF-α and particularly IL-1α were significantly higher than when the antigens were added alone.

Conclusion

The following conclusions may be drawn from part 2 of experiment 12;

1. Biologically active Nephronin can be synthesised using short chain acids.
2. pH adjustment of the final product seems to play a pivotal role in determining whether the preparations are biologically active. Although at present an explanation for this phenomenon is not possible, it is thought that since NaOH is a strong alkali, it may hydrolyse the Nephronin, rendering it ineffective in inhibiting the mitogen induced cytokine production.
3. Since only the preparations where their pH was adjusted to neutral with NaOH were tested against streptococcal antigens, and given the fact that such preparations were ineffective in inhibiting LPS induced cytokine production, confirms that these preparations are also ineffective in inhibiting streptococcal mitogen induced cytokine production.

Preparations of Nephronin that are effective in inhibiting LPS-induced cytokine production, should be tested for their effect on streptococcal mitogen induced cytokine preparations.

This experiment also demonstrates for the first time that Nephronin can be synthesised using acids with short carbon chain length.

Experiment 13

Experiment 13 was performed in order to optimise the synthesis of Nephronin, however, since the data from experiment 12 was not available whilst the synthesis was being carried out, the pH of the final products were adjusted with NaOH. The results from experiment 12 have now shown that adjustment of pH with NaOH, results in an inactive product. As a result, the preparations from experiment 13 was not tested for their biological activity.

Experiment 14

The results from experiment 12 had shown that synthesis of a biologically active Nephronin, utilising POCl3 to activate acetic acid followed by addition to Na3Citrate and pH adjustment of the final product with ammonium hydroxide was possible. Furthermore, I have also been able to obtain some propanoic acid that I shall use in a similar synthesis procedure.

Aims

1. Whether varying the ratio of the reaction mixtures increased the potency of the synthesised Nephronin.
2. To test the effect of NaHCO3 on the potency of Nephronin, when used to pH the samples.

Methods

The following reaction mixtures were set up.

1. 450µl acetic acid + 3ml hexane + 150 µl POCl3 + 600mg Na3Citrae.
2. 230µl acetic acid + 3ml hexane + 370µl POCl3 + 300mg Na3Citrate.
3. 300µl propanoic acid + 3ml Hexane + 370µl POCl3 + 300mg Na3Citrate.
4. 300µl acetic acid + 3ml Hexane + 100µl POCl3 + 300mg Na3Citrate. (Repeat of the synthesis in experiment 12).

The POCl3 reaction mixture was allowed to proceed for 30min at RT prior to addition of Na3Citrate to each tube. The reaction was then allowed to proceed at RT over night.

It is very important to note that the tubes were capped under nitrogen at both stages.

The samples were F/D to remove the solvent. It is noteworthy that the liquid in the tubes did not freeze completely. The solvent was evaporated under vacuum. To each tube 1ml of Hexane was added, the tubes were mixed vigorously and the solvent evaporated under vacuum. To the dried samples 2ml of water was added, mixed vigorously to dissolve the material. The samples did not appear to be very water soluble. Once the samples were dissolved in water, they were F/D to remove any traces of solvent. The lyophilised samples were resuspended in 5ml of water. Because of the time taken for this process the samples were left at RT over night.

In each case the samples were divided into two. The pH of one half was adjusted to neutral with 10% ammonium hydroxide and the other half with a solution of 2M NaHCO3.

To test the bio-activity of each sample, either 10µl or 50µl of each sample was added to PBMC at 1.5x106 cells/ml containing 1ng/ml LPS. The cells were harvested at 24 and 72h post-stimulation and the concentration of TNF-α and IL-1α in the culture supernantants measured by sandwich ELISA.

To test the inhibition of PHA-stimulated cytokine production, 50µl of each sample was added to 1ml of PBMC at 1.5x106 cells/ml containing 5µg/ml of PHA.

Given the fact that certain preparations of Nephronin are also able to inhibit the stimulation of PBMC by PHA, it was imperative to demonstrate if Nephronin had any inhibitory effect on other stimulants that exerted their biological effect differently from those of LPS.

LPS exerts its biological activity by binding to a serum protein, LPS binding protein (LBP). This complex then binds to CD14 on the surface of macrophages and results in the stimulation of cells and release of cytokines.

Gram-positive bacterial toxin (superantigen) activates cells in an entirely different pathway to that of LPS. They activate lymphocytes by binding to the outer cleft of the major histocompatibility complex (MHC) and the T-cell receptor (TCR). Activation of these immune cells by the superantigens is TCR Vβ restricted.

Streptococcal membrane protein (M-protein) and entrotoxins Spe A and Spe B were used because of availability. Streptococcal entrotoxin Spe A and Spe B are shown to be superantigens.

50µl of each of the preparations were added to PBMC at 1.5x106 cells/ml containing either 50ng/ml of Spe A, Spe B or M-protein.

Results

It is important to point out that the portion of the samples that were pH adjusted with NaHCO3 did not show any inhibition of the cytokine production. It is noteworthy that in many cases, the amount of cytokine detected was higher than the cells stimulated with the appropriate stimulant alone.

Figs 1 and 2 clearly demonstrate that when Nephronin was prepared using a similar method as in experiment 12, a biologically active compound is produced. Preparation 1 is consistently able to inhibit the LPS induced TNF-α and IL-1α at 24 and 72h post stimulation. It is noteworthy that all preparations showed significant inhibition of IL-1α at 24h post stimulation, however, this effect is diminished by 72h for all samples, except preparation 1, although its potency is considerably reduced.

Figs 3 and 4 show the effect of different preparations of Nephronin on inhibition cytokine by PBMC, 24 hour post-stimulation with streptococcal superantigens Spe A, Spe B or bacterial membrane protein (M-protein). Once again, preparation 1 is able to consistently inhibit both TNF-α and IL-1α production.

Figs 5 and 6 show the effect of the same preparations of Nephronin, 72h post-stimulation of the PBMC. Interestingly the inhibition of the cytokine production observed by some of the preparations, in particular preparation 1 has diminished. Although at present no explanation is offered as to why Nephronin is able to inhibit superantigen stimulation of cells, it is thought that Nephronin may be able to dampen the immune response non-specifically. The fact that its effect is diminished over a 72 hour period may be due to up take and break down of Nephronin by the cells.

Conclusions

The data from experiment 14 demonstrates that;

1. Biologically active Nephronin can be repeatedly synthesised.
2. The ratio of acid and POCl3 seem to play an important role in producing active Nephronin.
3. Nephronin is also able to inhibit cytokine production by PBMC stimulated with other stimulants, such as streptococcal superantigens Spe A, Spe B and streptococcal membrane protein (M-protein).

Experiment 15

The synthesis of Nephronin is based on esterification of a fatty acid molecule to the hydroxyl group on the citrate molecule. However, since there are three carboxyl groups on the citrate molecule, it is possible that the presence of these groups results in reactions that consume a large portion of the acid chloride formed during the POCl3 reaction. In an attempt to increase the yield, it was postulated that if Na3Citrate was first mixed with the acid, in this instance, acetic acid or propanoic acid, it might be possible to increase the chances of the acid chloride formed more readily available to react with the correct hydroxyl group.

The following information was used to calculate the relative amount of each of the components.

	MW	s.g	vol
Na3Citrate	294.1		
POCl3	153.3	1.645	93
Acetic acid	60.05	1.049	57
Propanoic acid	74.05	0.993	74

Given the fact that there are three carboxyl groups on the citrate molecule, at least three fold excess of the relevant acid was used.

The following reaction tubes were set up

1. 300mg Na3Citrate + 2ml hexane + 180µl acetic acid
2. 300mg Na3Citrate + 2ml hexane + 230µl propanoic acid
3. 300mg Na3Citrate + 2ml hexane + 180µl acetic acid (control)
4. 300mg Na3Citrate + 2ml hexane + 230µl propanoic acid (control)

The following reactions were set up in order to test whether a nine-fold excess of each acid improves the potency of the Nephronin being produced.

5. 300mg Na3Citrate + 2ml Hexane + 540µl Acetic acid
6. 300mg Na3Citrate + 2ml Hexane + 690µl propanoic acid

The mixture of each tube was mixed and allowed to stand at RT over night.

The following day, the POCl3 reaction mixture for each tube was prepared as listed below. The ratio of acids were calculated on the bases that at this point there would be one hydroxyl group available for esterification

with acid chloride formed. Furthermore, since each molecule of POCl3 can theoretically convert three acid molecules to acid chlorides, the volume of POCl3 was reduced accordingly.

1. 2ml Hexane + 60µl acetic acid + 31µl POCl3
2. 2ml Hexane + 75µl propanoic acid + 31µl POCl3
3. 2ml Hexane + 60µl acetic acid (control)
4. 2ml Hexane + 75µl propanoic acid (control)
5. 2ml Hexane + 60µl acetic acid + 31µl POCl3
6. 2ml Hexane + 75µl propanoic acid + 31µl POCl3

The tubes were capped under nitrogen and the POCl3 reaction was allowed to proceed at RT for 30min prior to being added to the appropriate acid treated citrate samples. The tubes were capped under nitrogen and the reaction was allowed to proceed over night. At this stage the solvent was thought to contain some of the active material. To remove the solvent effectively, the preparations were F/D. 2ml of hexane was added to the lyophilised material, mixed vigorously and the solvent was evaporated under vacuum. The dried material was resuspended in water and the pH adjusted to neutral with a solution of 10% ammonium hydroxide. To remove any traces of the solvent, the samples were once again F/D. Finally, the lyophilised preparations were resuspended in water and the pH adjusted to neutral with ammonium hydroxide. The final volume was adjusted to 5ml, prior to filter sterilising the samples for biological studies.

Results

Figs 1-4 shows the effect of different preparations of Nephronin in inhibiting LPS-induced TNF-α and IL-1α production at 24 and 72h post-stimulation. Preparation 5 seems to be the most potent, inhibiting LPS-induced TNF-α production by 54% and IL-1α by 95% by 24h post stimulation. Preparation 1 shows the least potency, where as preparations 2 and 6 show similar potency. By 72h post-stimulation the potency of these preparations is considerably reduced, however, the pattern remains the same.

40µl of the control preparations 3 and 4 were tested for their effect. These samples did not show any inhibitory effect. It is noteworthy that these preparations caused a higher cytokine secretion by PBMC than LPS alone.

Fig 5 shows the effect of these preparations in inhibiting PHA induced TNF-α and Il-1α production.

All of the samples including the controls showed some inhibition of PHA induced cytokine production. However, the only preparations that showed a significant inhibition were preparations of Nephronin, samples 5 and 6. Interestingly, sample 6 is more potent in inhibiting TNF-α production, whereas preparation 5 inhibits IL-1α production totally at 24 post-stimulation. Once again, the potency of these preparations seem to have been reduced considerably by 72h post-stimulation, however, preparation 5 continues to inhibit TNF-α and IL-1α by 82 and 69% respectively.

Figs 6-9 show the effects of different preparations of Nephronin on stimulation of PBMC by streptococcal toxins, Spe A, Spe B and membrane protein (M protein). Interestingly, preparation 5 is the only sample that is

able to consistently inhibit both TNF-α and IL-1α. The effect is more pronounced at 24h post-stimulation, and is significantly diminished by 72h post-stimulation, particularly in the case of IL-1α secretion.

Conclusion

Data from experiment 15 clearly demonstrates that although Nephronin can be synthesised, production of potent Nephronin very much depends on the ratio of the reactive components used in the synthesis procedure. The fact that preparation 5 seems to be the most potent sample suggests that pre-treatment of Na3Citrate with the acid may be advantageous, however, this requires further optimisation.

There are four important conclusions that one may draw from these results.

1. The ratio of POCl3 used in these reactions is of particular importance. Data from experiment 14 suggested that excess of POCl3 used in the synthesis reaction resulted in the production of a less active compound. Interestingly, using less of POCl3 also produced the same effect. The ratio of POCl3 to acid needs to be optimised.
2. The control preparations 3 and 4 were totally ineffective in inhibiting LPS-induced cytokine production. They showed some effect in inhibiting the PHA-induced cytokine production, however, this is thought to be due to Na3Citrate concentration.
3. Biologically active Nephronin can also inhibit other immune stimulants in inducing cytokine production. Although at present an explanation for this effect is not offered, it is thought that Nephronin may be able to non-specifically dampen immune response.
4. These preliminary data also indicate that Nephronin may be synthesised from longer chain carboxylic acids, for example, Propanoic, Butyric acid, Hexanoic acid and Octanoic acid. However, it seems very important that the carboxylic acid used is liquid, and water miscible, rather than water immiscible.

Experiment 16

The data from experiment 15, clearly showed that the ratio of POCl3 played an important role in producing a potent version of Nephronin. Furthermore, the pre-treatment of Na3Citrate with the relevant acid had a positive effect on the final product. It is important to note that when a nine-fold excess of the acid was used, the synthesised material was more potent.

In this experiment it was decided to repeat the synthesis of experiment 12 and possibly use this preparation as a standard for comparison between various preparations.

The following reaction mixtures were set up;

1. 300mg Na3Citrate + 2ml Hexane + 540µl of acetic acid.
2. 300mg Na3Citrate + 2ml Hexane + 690µl of propanoic acid.
3. 300mg Na3Citrate + 2ml Hexane + 540µl of acetic acid.
4. 300mg Na3Citrate + 2ml Hexane + 690µl of propanoic acid.

The tubes were capped under nitrogen and incubated at RT for 4h with mixing on a roller.

The POCL3 reactions were set up as below, where an approximate 4 fold of the acid was used.

1. 2ml Hexane +270µl Acetic Acid + 100µl POCl3
2. 2ml Hexane +295µl Propanoic Acid + 100µl POCl3
3. 2ml Hexane +270µl Acetic Acid (no POCl3 added, Control)
4. 2ml Hexane +295µl Propanoic Acid (no POCl3 added, Control)

The tubes were capped under nitrogen and after 30 minutes incubation at RT with mixing on a roller. After this time, the POCl3 reaction mixtures were added to the appropriate tubes set up for Na3Citrate treatment.

Repeat of the synthesis performed similarly to experiment 12.

The following POCl3 reactions were set up.

5. 300mg Acetic acid + 3ml Hexane + 100µl POCl3
6. 300mg Acetic acid + 3ml Hexane (no POCl3 was added, Control).

The tubes were capped under nitrogen, incubated at RT with mixing on a roller for 20minutes. After this time, 300mg of Na3Citrate was weighed and added to each tube. Each tube was capped under nitrogen and incubated at RT, over night.

Whilst this experiment was being performed, the possibility that Nephronin may be formed as a solvent soluble material, after addition of the POCl3 reaction mixture to the treated Na3Citrate was considered. To test this hypothesis, the following reaction tubes were set up.

7. 300mg Na3Citrate +540µl Acetic acid.
8. 300mg Na3Citrate + 690µl Propanoic acid

The citrate and the acids were mixed very vigorously. The Na3Citrate formed a very hard mass with acetic acid that adhered to the bottom of the tube. In the case of propanoic acid, the Na3Citrate seemed to remain as crystalline during the time of incubation. After 2h incubation at RT, 4ml of hexane were added to each tube. The contents of the tubes were mixed very vigorously ensuring full mixing of Na3Citrate with the acids. The tubes were then incubated at RT over night. The solvent was then decanted into appropriately labelled tubes.

The following POCl3 reaction mixtures were set up,

7'. 2ml Hexane + 270µl Acetic acid + 100µl POCl3.

8'. 2ml Hexane + 900µl Propanoic acid + 100µl POCl3

The tubes were capped under nitrogen and incubated at RT for 30min with mixing on a roller.

The reaction mixtures were then added to the solvent decanted from the Na3Citrate treatment tubes 7 & 8. The tubes were capped under nitrogen and the content mixed and incubated at RT with continuous mixing on a roller, for only 2h. To test the solvents soluble material, the solvents were decanted into marked tubes and freeze-dried.

All reaction mixtures were dried under vacuum. 2ml of hexane was added to each tube and re-dried under vacuum.

In each case, the lyophilised material was resuspended in 3ml of water. The pH adjusted to neutral with a solution of 10% ammonium hydroxide. The samples were freeze-dried and the lyophilised material resuspended in water and pH adjusted to neutral with ammonium hydroxide. On this occasion, 400µl of 10 x PBS was added to each tube and the final volume of each sample adjusted to 4ml. All samples were filter sterilised using a 0.2µ membrane prior to testing for their biological activity.

Results

In this experiment preparations 3, 4 and 6 were produced without the use of POCl3. These samples are to be used as controls for the relevant preparations. Consequently, they were only used at the highest concentrations that the test samples were used.

Figs 1 and 2 show the dose dependent inhibition of LPS-induced TNF-α and IL-1α. These new preparations show much higher levels of inhibition compared to preparations in experiment 15. These findings confirm that the ratio of POCl3 is very important in producing more potent preparations of Nephronin. Interestingly, these samples caused slightly higher concentrations of TNF-α production when only 10µl of the preparations were tested.

It is also noteworthy that preparation 2, where propanoic acid was used in producing Nephronin, was able to inhibit LPS-induced IL-1α production more significantly than preparations where acetic acid was used.

Figs 3 and 4 show the inhibition of cytokine production by the same preparations as above at 48h post-stimulation. Preparations 1 and 2 showed a similar pattern of inhibition as in 24h post-stimulation. Preparation 5, which was essentially a repeat preparation of experiment 12, had become less potent by 48h post-stimulation. Interestingly, the pattern for LPS-induced IL-1α production is very different from that of 24h post-stimulation. Preparation 5 seems to be ineffective in inhibiting IL-1α production. Indeed, an increase in the concentration of IL-1α concentration was detected when lower amounts of this preparation were used. This may be due to the fact that preparation 5 contains material that is toxic to the cells and Nephronin is able to exert its inhibitory effect, only when an increased volume of the sample is used.

A number of preparations were made, both as control and also to test whether active Nephronin can be produced by using the solvent soluble material after treatment of Na3Citrate with the acid in hexane. Preparations 3, 4 and 5 are controls for preparations 1, 2 and 5 respectively. In these preparations, the same procedure for synthesising Nephronin was used except that POCl3 was omitted.

Preparations 7 and 8 are the solvent soluble material from pre-treatment of Na3Citrate by acetic acid and propanoic acid respectively. In these preparations the solvent soluble material was put through the same procedure as that for Nephronin.

Figs 5 and 6 show the effect of eight different preparations on LPS induced TNF-α and IL-1α, 24h post-stimulation of PBMC. In each case 40μl of each preparation was tested. Preparations 1, 2 and 5 inhibited TNF-α by 63, 66 and 51% respectively. These same samples showed 100% inhibition of IL-1α at these concentrations. Control preparations 3, 4 and 6 showed 21, 21 and 10% inhibition of TNF-α production. Interestingly, these same preparations were more effective in inhibiting IL-1α production. Although at present no explanation can be offered for these apparent inhibitions, these effects are thought to be due to Na3Citrate. Consequently, when data are being analysed, such effects should be taken into consideration.

Figs 7 and 8 show these same preparations 48h post-stimulation. Preparations 1, 2 and 5 were able to inhibit TNF-α production by 59, 66 and 33%, whereas only preparations 1 and 2 inhibited IL-1α production by 78 and 87% respectively. Control preparations 3, 4 and 6 marginally inhibited TNF-α, where as the concentration of IL-1α production was higher than cells treated with LPS alone.

Preparations 7 and 8 did not show any inhibition of TNF-α and IL-α. The increase in the levels of cytokine production is though to be due to the toxic effects that these preparations may have on PBMC.

The effects of these same samples were also tested on the PHA-induced cytokine production. Preparations 1, 2 and 5 were potent inhibitors of PHA-induced TNF-α at 24 and 48h post-stimulation. Interestingly, control preparations 3 and 6 also showed significant inhibition. A similar pattern of inhibition was also observed for IL-1α, except that at 48h post-stimulation, control preparation 4 also showed significant inhibition of IL-1α.

Preparations 7 and 8 were ineffective and did not show any significant variance from cells treated with PHA only.

Conclusion

The following conclusions can be drawn from the data obtained in experiment 16.

1. The ratio of POCl3 to acid is very important in producing more potent Nephronin.
2. Pre-treatment of Na3citrate with the appropriate acid, results in production of more potent Nephronin.
3. The solvent soluble material contains toxic material resulting in further activation of cells and production of more cytokine production.
4. Some of the biological activities observed are due to Na3Citrate, particularly in the case of PHA-induced cytokine production. However, Nephronin remains significantly more potent than the control preparations.

Experiment 17

Aims

Butyric acid has just been received, which will be used in preparing Nephronin.

1. To test the hypothesis, that an active compound can also be synthesised by acids of longer carbon chains, acetic acid, propanoic acid and butyric acid were used in the synthesis of Nephronin.
2. To test whether increasing carbon chain length of the acid, has an effect on the potency of the synthesised Nephronin, dose dependent inhibition of LPS induced cytokine production for these preparations are to be examined.
3. To test whether increasing the ratio of acid to Na3Citrae in the pre-treatment of citrate would result in the production of more potent products the molar ratio of the acid to citrate is to be increased to 10:1 fold excess acid.

4. To examine the possibility that Na3Citrate treated with the acid might result in an acid soluble material that might result in a more potent preparation of Nephronin, the acid soluble material was used in the synthesis procedure.

Method

Based on previous experiments, the following reactions were set up.

1. 300mg Na3Citrate + 540µl of Acetic Acid
2. 300mg Na3Citrate + 540µl of Acetic Acid (sup taken)
3. 300mg Na3Citrate + 690µl of Propanoic Acid
4. 300mg Na3Citrate + 690µl of Propanoic Acid (sup takien)
5. 300mg Na3Citrate + 835µl of Butyric Acid
6. 300mg Na3Citrate + 835µl of Butyric Acid (sup taken)

Na3Citrate was mixed with the acid for 2h at RT. In the case of acetic acid, the Na3Citrate solidified forming one mass that strongly adhered to the bottom of the tube. After 2h incubation, the liquid portion from each tube was decanted. In the case of tubes 2, 4 and 6, the liquids were placed in appropriately numbered tubes. To the tubes 1-6, 2 ml of hexane was added and mixed vigorously. The corresponding treated Na3Citrate (the solids) from tubes 2, 4 and 6 were then numbered 7, 8 and 9 respectively.

The following POCl3 reactions were set up for these reactions.

1. 2ml Hexane + 180µl Acetic acid + 100µl POCl3
2. 2ml Hexane + 180µl Acetic acid + 100µl POCl3
3. 2ml Hexane + 230µl Propanoic acid + 100µl POCl3
4. 2ml Hexane + 230µl Propanoic acid + 100µl POCl3
5. 2ml Hexane + 250µl Butyric acid + 100µl POCl3
6. 2ml Hexane + 250µl Butyric acid + 100µl POCl3
7. 2ml Hexane + 180µl Acetic acid + 100µl POCl3
8. 2ml Hexane + 230µl Propanoic acid + 100µl POCl3
9. 2ml Hexane + 250µl Butyric acid + 100µl POCl3

Note. Hexane was not added to the solids in tubes.7, 8 and 9, until the POCl3 reaction mixture was added to these tubes.

The POCl3 reaction tubes were capped under nitrogen. The tubes were placed on a roller to allow mixing and after 30 minutes incubation at RT, the contents of each tube was poured into the corresponding tubes. These tubes were also capped under nitrogen. The tubes were mixed vigorously, so that the contents of each tube were thoroughly mixed with the POCl3 reaction mixture.

The reaction tubes 1-6 were placed on a roller and incubated for 2h at RT. The tubes were then freeze-dried. Reaction tubes 7-9 were incubated on a roller over night at RT.

To evaporate the solvent, the reaction tubes were frozen on dry ice and the contents freeze-dried. 2 ml of hexane was added to the freeze-dried material. The tubes were mixed vigorously and the hexane evaporated under vacuum. The dried material was resuspended in water and the pH adjusted to neutral with 10% ammonium hydroxide. The samples were then freeze-dried. Finally, the lyophilised material was resuspended in water, and in each case the pH adjusted to pH 7.2-7.4. The volume of these samples was then adjusted to 5ml and the samples were filter sterilised prior to testing for their biological activities.

Results

Figs 1 and 2 show the effect of Nephronin in inhibiting LPS-induced TNF-α and IL-1α. The inhibition profile shows a clear dose dependent response to increasing volumes of different preparations of Nephronin. Interestingly, increasing carbon chain of the acid used, seems to increase the potency of these preparations for both cytokines measured. In these preparations Na3Citrate was first mixed with the appropriate acid where a ten fold molar ratio of the acid to the Na3Citrate was used. These preparations show a much higher potency in inhibiting LPS-induced cytokine production than previous preparations.

Figs, 3-8, show the comparison of biological activity of preparations where the acid treated Na3Citrate mixture and the acid soluble material were used in the synthesis procedure.

Figs 3 and 4 show the inhibition of LPS-induced TNF-α and IL-1α, where acetic acid was used in the synthesis procedure. Clearly the acetic acid soluble material did not result in a compound that could inhibit TNF-α and IL-1α production. Rather, the data shows an increase in the levels of cytokines measured, suggesting that this particular preparation may be toxic to the cells.

Figs 5 and 6 show the inhibition of LPS-induced TNF-α and IL-1α, where propanoic acid was used in the synthesis procedure. Interestingly, where propanoic acid soluble material was used, the resulting product was very potent in inhibiting both TNF-α and IL-1α production. However, the potency of the acid soluble material seems to be slightly less than the preparation where the acid treated mixture was used. The difference in their potency is more pronounced for IL-1α than TNF-α.

Figs 7 and 8 show the inhibition of LPS-induced TNF-α and IL-1α, where butyric acid was used in the synthesis procedure. The data obtained from the dose dependent inhibition of these cytokines is very

similar to that for propanoic acid. Interestingly, the acid soluble material seems to be very potent in inhibiting LPS-induced cytokine production at low concentrations. However, the inhibition profile seems to plateau at higher concentrations of this preparation for TNF-α. Both preparations are extremely potent in inhibiting IL-1α production, where 100% inhibition is achieved when 10μl of either preparation is used.

This difference in potency observed between preparations for propanoic acid and butyric acid may be due to the amount of active material that may be present in each preparation. Alternatively, this effect may be due to the concentration of Na3Citrate present in preparations where the whole mixture was used. Data from previous experiments clearly demonstrates some inhibition of cytokine production that is ascribable to citrate salt.

Figs 9 and 10 show the dose dependent inhibition of LPS-induced TNF-α and IL-1α for preparations of Nephronin, where the Na3Citrate was first treated with the appropriate acids, followed by removal of the acid soluble material, (preparations 7-9). The inhibition profile for these three preparations shows an increase in the potency of the preparations with increasing carbon chain length of the acids used. This increase in the potency is more pronounced for IL-1α, although the trend is true for both cytokines.

Figs 11 and 12 show the effect of all nine preparations on PHA-induced TNF-α and Il-1α production. The data are expressed as percent inhibition of the cytokines produced compared to cells stimulated with PHA alone. Preparations 1, 3 and 5 inhibited PHA-induced TNF-α production by 75, 58 and 60% respectively. These preparations correspond to preparations where acetic acid, propanoic acid and butyric acid were first added to the Na3Citrate and the mixture was used in the synthesis procedure. It is noteworthy that with increasing carbon chain length of the acid, the synthesised material became less potent in inhibiting PHA-induced TNF-α. Considering that the same amount of Na3Citrate was used in each, the reduction in inhibition must be inversely proportional to the increasing carbon number. It is important that under the test conditions, such a trend was not observed for inhibition of IL-1α. This is not surprising, since almost 100% inhibition was observed when 40μl of each sample was used.

Preparations 7, 8 and 9, correspond to preparations where Na3Citrate after removal of the acid soluble material from treatment with acetic acid, propanoic acid and butyric acid respectively, were used in the synthesis procedure. These preparations inhibited PHA-induced TNF-α production by 74, 81 and 67% respectively. These preparations were more potent compared to their corresponding preparations 1, 3 and 5 in inhibiting TNF-α production. Nevertheless, a similar pattern of inhibition was observed as above with an exception for preparation 8. These preparations exhibited 100% inhibition of PHA-induced IL-1α, and a distinguishing inhibition pattern was not observed.

Preparations 2, 4 and 6, corresponds to preparations where the acid soluble material decanted from treatment of Na3Citrate with acetic acid, propanoic acid and butyric acid respectively, were used in the synthesis procedure, showed 37, 22 and 37% inhibition PHA-induced TNF-α production. Interestingly, these preparations only showed 2, 1 and 25% inhibition of PHA-induced IL-1α production.

Conclusions

Several conclusions can be drawn from the data obtained in this experiment.

1. Biologically active Nephronin can also be synthesised with acids of longer chain length than acetic acid. This is important, since initial attempts with palmitic, Oleic, pentadecanoic acid etc, resulted in a biologically active pentadecanoic acid preparation. These findings suggest that, theoretically at least, Nephronin can be synthesised with acids with carbon chain length of up to 14 carbon (pentadecanoic acid). Attempts should be made to synthesis Nephronin from saturated and branched forms of these acids. This might result in preparations that have different biological activities. However, this does not discount the fact that fatty acids of longer carbon chain can also be used in the synthesis process. Although, the procedure may need to be optimised.
2. Using acids with increasing carbon chain length results in compounds that are more potent than preparations with acids of lower carbon chain length.
3. It is also possible to synthesis a compound using the acid soluble material that can inhibit LPS-induced TNF-α and IL-1α production. The potency of these preparations increased with the carbon chain length of the acids used.
4. Preparations where acid soluble material was used in the synthesis of Nephronin were considerably less potent in inhibiting TNF-α production compared to their ability in inhibiting IL-1α for PHA-induced cytokine production. This is particularly important in the cases of propanoic acid and butyric acid, where LPS-induced cytokine production was also inhibited significantly.
5. Treatment of the Na3Citrate with the relevant acid, followed by removal of the acid soluble material resulted in preparations that were more potent than any other preparation to date.
6. Increasing the ratio of the acid to Na3Citrate to 10:1, in general resulted in more potent preparations.
7. Synthesis of active Nephronin can be achieved in only 2h, when the POCl3 reaction mixture is added to the citrate. The time of synthesis may play an important role in final optimisation of the synthesis procedure.

Because of the importance of the data obtained from this particular experiment, a more comprehensive testing of these samples was performed. The effect of these samples were tested at 24 and 48h post-stimulation.

Repeat of Experiment 17 (Experiment 17R)

The preparations from experiment 17 were used to re-evaluate the activities obtained for each sample.

Results

Figs 1-4 from experiment 17R show the dose dependent LPS-induced inhibition of TNF-α and IL-1α production by preparations of Nephronin where acetic acid was used in the synthesis procedure.

Figs 1 and 2 show the inhibition of TNF-α by three preparations of Nephronin at 24 and 48h post-stimulation.

Preparation 1 refers to the product where Na3Citrate was first treated with acetic acid, and the mixture was used in the synthesis procedure, inhibited TNF-α by 41% at 24h and only 18% at 48h.

Preparation 2 refers to the product where the acid soluble material after treatment of Na3Citrate with acetic acid was used in the synthesis procedure, showed minimal inhibition of TNF-α at 24 and 48h post-stimulation. Indeed the data show an increase in the concentration of TNF-α compared to cells stimulated with LPS alone. This might be due to toxic effects of this preparation.

The inhibition profile for preparation 7, where Na3citrate was first treated with acetic acid and after removing the acid soluble material, the remaining solid was used in the synthesis procedure, showed a more potent inhibition. The apparent inhibition of LPS induced TNF-α was considerably reduced by 48h post-stimulation from 57% to 30%. The reduction in the potency of these preparations at 48h post-stimulation might be due to up take of Nephronin by the cells.

Figs 3 and 4 show the inhibition profile of IL-1α for the same samples. A similar inhibition profile is produced for IL-1α, except that preparations 1 and 7 inhibited LPS-induced IL-1α by 94 and 87% respectively at 24h post-stimulation. This inhibition is reduced to 15 and 22% by 48h post-stimulation. These preparations seem to be more potent in inhibiting IL-1α than TNF-α. Preparation 2 did not show any significant inhibition of either TNF-α or IL-1α at both time points.

Figs 5-8 show the inhibition profile of LPS-induced TNF-α and IL-1α for preparations of Nephronin, where propanoic acid was used in the synthesis procedure. Preparation 3 refers to the mixture of acid and Na3Citrate, preparation 4, the acid soluble and preparation 8 the Na3Citrate after removal of acid soluble material, that were used in the synthesis procedure. The dose dependent profiles are very similar

to the acetic acid preparation, except that the acid soluble material could inhibit TNF-α at both 24 and 48h post-stimulation, with the potency reduced by 48h.

In the case of IL-1α inhibition, all three samples showed inhibition of IL-1α at 24h post-stimulation. Interestingly, preparation 4, where the acid soluble material was used, 27% inhibition was achieved. By 48h post-stimulation the profile of IL-1α had changed so that none of the three samples showed any inhibition, in fact an increased level of IL-1α was measured, suggesting stimulation of the PBMC.

Figs 9-12 show the inhibition profile of LPS-induced TNF-α and IL-1α for preparations where butyric acid was used in the synthesis procedure. Preparation 5 refers to acid treated Na3Citrate, where the mixture was used in the synthesis. In preparation 6, the acid soluble material was used and in preparation 9, the Na3Citrate after removal of the acid soluble material was used in the synthesis procedure. These preparations essentially gave the same profile as before, except that they seem to be more potent in inhibiting both TNF-α and IL-α production. In the case of TNF-α, they remained potent at 24 and 48h post-stimulation. However, in the case of IL-1α, preparations 5 and 9 achieved 100% inhibition with only 10μl of each sample, whereas preparation 6 showed 40% inhibition. Interestingly, preparations 5 and 9 remained very potent and achieved 100% inhibition when 20μl of the samples was used. This suggests that Nephronin binds to the cells and is being used up with time. However, preparation 6 did not show any inhibition at 48h post-stimulation, and seemed to stimulate the cells since the concentration of IL-1α was higher than cells with LPS alone.

Next, the inhibition profile of these samples for PHA-induced TNF-α and IL-1α was investigated.

Figs 13-16 shows the inhibition profile for Nephronin preparations where acetic acid was used as above. All three preparations showed inhibition of PHA-induced TNF-α. Interestingly, they continued to inhibit TNF-α production by 48h post-stimulation with increased potency. Similar results were also obtained for PHA-induced IL-1α production. Once again, the inhibition profiles show an increase in the amount of inhibition at 48h than 24h post-stimulation.

Figs 17-20 show the inhibition profiles for preparations of Nephronin where propanoic acid was used. Inhibition profiles for these preparations are very similar to those previously shown, where similarly at 48h post-stimulation they seem to be more potent than at 24h.

Figs 21-24 shows the PHA induced inhibition of cytokine production, where propanoic acid was used. Once again the inhibition profile for these preparations are very similar to the previous two preparations. Similarly, these samples also seem to increase in their potency by 48h post-stimulation.

Conclusions

The data obtained from this experiment is very similar to that obtained from experiment 17. However, the fact that these samples were also studied at 48h post-stimulation enabled the following conclusions to be drawn from these results.

1. The ability of Nephronin to inhibit TNF-α and IL-1α is reduced with time, suggesting that Nephronin might be taken up by cells and degraded. Given the fact that if Nephronin were to bind to the mitogen itself and neutralise its effect, the incubation time post-stimulation should not effect the outcome. Nevertheless, it should also be born in mind that cytokines such as TNF-α and IL-1α stimulate the release of other cytokines as well as stimulating the release of more of the same. Clearly further studies are required to demonstrate the mechanism of the action of Nephronin.
2. Although the acid soluble material used in the synthesis of Nephronin were significantly less potent in inhibiting LPS-Induced cytokine production, they seem to be very potent in inhibiting PHA-induced cytokine production. Interestingly, this potency increases with the time of incubation post-stimulation.
3. Since the acid soluble material contains very little Na3Citrate, the observed inhibitions can not be ascribed to the citrate salt.

Experiment 18

Results from experiment 16 showed that when Na3Citrate was mixed with hexane and the acid, the solvent soluble material was not effective in inhibiting LPS-induced cytokine production. In fact these samples seem to cause an increase in the amount of cytokines measured.

Aims

1. To test whether removal of the solvent soluble material effects the potency of the final product. Data from experiment 16 suggested that the solvent soluble material was toxic to the cells. Consequently, removal of this fraction might assist in removing some of the toxic compounds that may be present in these preparations.

Methods

The following reaction tubes were set up

1. 300mg of Na3Citrate + 540µl of Acetic acid + 2ml Hexane
2. 300mg of Na3Citrate + 690µl of Propanoic acid + 2ml Hexane
3. 300mg of Na3Citrate + 835µl of Butyric acid + 2ml Hexane

4. 300mg of Na3Citrate + 540µl of Acetic acid + 2ml Hexane
5. 300mg of Na3Citrate + 690µl of Propanoic acid + 2ml Hexane
6. 300mg of Na3Citrate + 835µl of Butyric acid + 2ml Hexane

After 4h incubation at RT, all samples were centrifuged at 4000rpm for 10 minutes. In the case of tubes 1, 2 and 3, the solvent layer was removed and the tubes were allowed to stand under in a fume hood for excess solvent to evaporate.

The following POCl3 reaction tubes were set up,

1. 2ml Hexane + 180µl Acetic acid + 100µl POCL3
2. 2ml Hexane + 230µl Propanoic acid + 100µl POCL3
3. 2ml Hexane + 250µl Butyric acid + 100µl POCL3
4. 2ml Hexane + 180µl Acetic acid + 100µl POCL3
5. 2ml Hexane + 230µl Propanoic acid + 100µl POCL3
6. 2ml Hexane + 250µl Butyric acid + 100µl POCL3

The tubes were capped under nitrogen and allowed to mix at RT on a roller for 25 minutes. After this time, the reaction mixtures were then added to the appropriate tubes containing the Na3Citrate. Once again the tubes were capped under nitrogen and incubated over night with mixing on roller.

The tubes containing the reaction mixtures were freeze-dried to evaporate the solvent. 2ml of Hexane was added to the lyophilised material and the content mixed vigorously. To remove the hexane the tubes were dried under vacuum. The lyophilised material was resuspended in 3ml of water and the pH adjusted to neutral with 10% ammonium hydroxide. The samples did not seem very soluble in water, however, addition of ammonium hydroxide to the solution seemed to assist solubilization of the samples. Upon addition of the ammonium hydroxide to the samples, a white fume was given off.

The pH of the samples was adjusted to pH 7.4 and the samples were freeze-dried. The lyophilised material was resuspended in 5ml of water and the pH re-adjusted to pH 7.4 as before. All preparations were filter sterilised prior to being tested for their biological activity.

Results

To evaluate whether removal of the solvent soluble material would enable cleaner preparations, three identical synthesis procedures for acetic acid, propanoic acid and butyric acid were performed. The only variance introduced was removal of the solvent soluble material from one of the synthesis during the synthesis procedure. Consequently, the solvent soluble material was removed from the duplicate preparations, 1, 3 and 5.

Figs 1-4 show the dose dependent inhibition of two preparations of Nephronin where acetic acid was used in the synthesis procedure. The inhibition profiles of these samples were tested at 24 and 48h post-stimulation.

Figs 1 and 3 show the inhibition of LPS-induced TNF-α production at 24 and 48h post-stimulation respectively. The inhibition curves are remarkably similar in both cases, except that the amount of TNF-α measured is slightly less at 48h post-stimulation. Figs 2 and 4 show the inhibition of LPS-induced IL-1α secretion. Interestingly these samples show good inhibition of IL-1α at 24h post-stimulation, which is considerably reduced by 48h post-stimulation. Both preparations show very close dose response curves.

Figs 5-8 show the inhibition profiles of preparations 2 and 5, where propanoic acid was used in the synthesis procedure. Once again a very similar profile was obtained for both samples. It is noteworthy that these particular preparations were not as potent as expected. It is likely that during the synthesis procedure minor adjustments were inadvertently introduced that effected the final product.

Figs 9-12 show the inhibition profile of preparations where butyric acid was used in the synthesis procedure. These preparations also produced almost parallel inhibition profiles. It is noteworthy that in the case of LPS-induced IL-1α production at 48h post-stimulation, the amount of IL-1α detected in the cells with LPS alone was considerably less than expected. However, the inhibition profiles obtained are very similar.

Given the fact that these preparations gave such close inhibition profiles, the dose response curves for PHA-induced TNF-α and IL-1α is shown for all six preparations.

Figs 13 and 14 show the inhibition of PHA-induced TNF-α and IL-1α at 24h and figs 15 and 16 for 48h post-stimulation. These preparations were very potent in inhibiting IL-1α production at both incubation times. However, preparations 2 and 5, were slightly less effective, which might be due to problems in the synthesis procedure. It is also noteworthy that at 48h post-stimulation, these samples showed reduced potency. This might be due to up take of Nephronin by the cells.

Conclusions

The data from this experiment suggests that removal of the solvent soluble material does not effect the potency of the final product. This might be due to the fact that much of the toxic material is removed during the freeze-drying process, including the solvent and some of the ammonium salt that are evaporable.

Experiment 19

Preparations 7, 8 and 9 from experiment 17 are the most potent samples produced to date. It was decided to repeat these synthesis. Furthermore, minor modifications were introduced in an attempt to optimise the synthesis reaction.

The following reactions were set up

1. 300mg Na3Citrate + 540µl Acetic acid
2. 300mg Na3Citrate + 690µl Propanoic acid
3. 300mg Na3Citrate + 835µl Butyric acid
4. 300mg Na3Citrate + 540µl Acetic acid
5. 300mg Na3Citrate + 690µl Propanoic acid
6. 300mg Na3Citrate + 835µl Butyric acid
7. 300mg Na3Citrate + 540µl Acetic acid
8. 300mg Na3Citrate + 690µl Propanoic acid
9. 300mg Na3Citrate + 835µl Butyric acid

When the appropriate acids were added to the tubes, the content was mixed vigorously. In the case of acetic acid treatment of Na3Citrate, the acid became very viscous.

After 2h incubation at RT, tubes 4, 5 and 6 were centrifuged at 4000 rpm for 10 minutes and the liquid was removed. Once again the tube containing the acetic acid was very viscous. After removal of the acid, the same volume as the starting volume of each acid was added to the appropriate tubes. After a further 3h incubation at RT, all tubes were centrifuged at 4000 rpm for 10 minutes and the liquid removed. The tubes were placed in fume hood with open caps to allow evaporation of excess acid.

The following POCl3 reactions were set up and after 30 minutes incubation at RT, the content of each tube was poured into the appropriate pre-treated citrate tubes.

1. 180µl Acetic acid + 2ml hexane + 100µl POCl3
2. 230µl Propanoic acid + 2ml hexane + 100µl POCl3
3. 250µl Butyric acid + 2ml hexane + 100µl POCl3
4. 180µl Acetic acid + 2ml hexane + 100µl POCl3
5. 230µl Propanoic acid + 2ml hexane + 100µl POCl3
6. 250µl Butyric acid + 2ml hexane + 100µl POCl3
7. 180µl Acetic acid + 2ml hexane + 100µl POCl3
8. 230µl Propanoic acid + 2ml hexane + 100µl POCl3
9. 250µl Butyric acid + 2ml hexane + 100µl POCl3

Two very important modifications were introduced in these preparations

1. In this experiment, the POCl3 reactions were not capped under nitrogen.

2. After addition of POCl3 to the pre-treated citrate tubes, once again the tubes were not capped under nitrogen.

In each case, the content of each tube was mixed vigorously to ensure full mixing of the reaction mixtures. The reaction tubes were incubated at RT over night with mixing on roller.

The solvent (the liquid phase) from tubes 7, 8 and 9 was removed. The tubes were placed in a fume hood to allow evaporation of the remaining amount of solvent. 2 ml of Hexane was added to each tube and the content of each tube mixed vigorously. The solvent was removed and once again the tubes were placed open in a fume hood to allow evaporation of the remaining amount of the solvent.

In the case of reaction tubes 1-6, the content of each tube including the solvent was freeze-dried. 2ml of hexane was added to the lyophilised material and after vigorous mixing of the tubes, the solvent was evaporated under vacuum.

In all cases 3ml of water was added to each tube. The solids did not dissolve very easily in water. However, addition of 10% ammonium hydroxide seemed to assist in the dissolving of the solids. The pH of each sample was adjusted to pH 7.4 and the samples were freeze-dried.

In order to gauge the concentration of the samples, the weight of preparation in each tube was determined.

Tube no	Weight in mg
1	620mg
2	730mg
3	860mg
4	488mg
5	730mg
6	960mg
7	700mg
8	700mg
9	700mg

The lyophilised material was resuspended in water and the pH adjusted to pH 7.4 with ammonium hydroxide. The final volume in each case was adjusted to give 100mg of sample per ml of water.

Results

The fact that none of these preparations were effective in inhibiting either LPS or PHA induced cytokine production, suggests that the synthesis had not been successful. The only modification of any importance that was introduced was to perform the reactions in air. This matter was brought to the attention of a chemist who suggested that esterification of Na3Citrate with the acid chloride did not require nitrogen and that these results may be false negative.

The fact that these preparations were ineffective in inhibiting mitogen induced cytokine production serves as a good control for these studies, in that only successful synthesis of Nephronin results in preparations that are able to inhibit both LPS and PHA stimulation. However, these particular synthesis need to be repeated and the results confirmed.

Experiment 20

In this experiment essentially the same procedure as experiment 19 was followed except that the amount of each reactant was increased by 10. The reactions were therefore performed in 50ml falcon tubes. The following reaction mixtures were set up.

1. 3g Na3Citrate + 5.4 ml Acetic acid
2. 3g Na3Citrate + 6.9ml Propanoic acid
3. 3g Na3Citrate + 8.35ml of Butyric acid.

The content of each tube was mixed thoroughly and the tubes were allowed to stand at RT with occasional mixing. After approximately 3h incubation, the tubes were centrifuged at 4000 rpm for 10 minutes and the acid product (liquid) was removed, the same volume of fresh acid was then added to each tube. The content of each tube was thoroughly mixed and allowed to stand at RT over night. The following day, the tubes were centrifuged at 4000 rpm for 5 minutes and the liquid was decanted. The tubes were placed in a fume hood, left open to allow evaporation of excess acid for 30-45 minutes.

The following POCl3 reaction mixtures were set up;

1. 1.8ml of Acetic acid + 10 ml Hexane + 1ml POCl3
2. 2.3ml of Propanoic acid + 10 ml Hexane + 1ml POCl3
3. 2.5ml of Butyric acid + 10 ml Hexane + 1ml POCl3

Once again the tubes <u>were not</u> capped under nitrogen to test the effect of air in the synthesis. The content of the tubes were mixed and placed on roller for 30 minutes. After this incubation period, the content of each tube was poured into the appropriate tubes containing the treated Na3Citrate tubes.

Again, the tubes **were not** capped under nitrogen. The content of each tube was mixed and the reaction tubes were incubated over night at RT.

After the POCl3 reaction, the solvent (liquid) from each tube was removed. 5ml of hexane were added to the remaining solids. The tubes were mixed vigorously and the hexane removed. The solids were once again washed with 5ml of hexane and the solids were allowed to dry in a fume hood.

Data from experiment 19 had clearly shown that the final lyophilised products were significantly heavier than the starting weight of the citrate. For this reason the weight of each sample was determined at this stage.

Samples	Weight g
1. Acetic acid reaction	3.58g
2. Propanoic acid reaction	5.1g
3. Butyric acid reaction	5.57g

The reason that the solid from the acetic acid reaction weighs so much less is thought to be due to some of the Na3Citrate dissolving in the acid and being removed when the acid (liquid) was removed. However, the lyophilised products weighed significantly more than the 3g starting weight of the Na3Citrate.

To each preparation 10ml of water was added To assist dissolving of the solids, ammonium hydroxide was added to each tube. Once again, a white fume was visible upon addition of ammonium hydroxide to each tube. This is thought to be ammonium chloride. The pH of the samples was adjusted to pH 7.4. The volume of each sample had increased to approximately 20ml. Each sample was divided into two, and freeze-dried. Because of the high concentration of salts, the samples regularly thawed out. However, after a prolonged freeze-drying process the weight of the lyophilised samples were determined.

1. Acetic acid preparation	4.11g
2. Propanoic acid preparation	6.116
3. Butyric acid preparation	6.79

Once again the weight of the samples had increased. This is presumably due to the weight of the ammonium salt of the samples.

The samples were dissolved in water and the pH adjusted to pH 7.4. The final volume of preparation was adjusted so that it contained 0.3g material /ml of water. The samples were filter sterilised as in previous experiments and tested on PBMC for their biological activity.

Results

These samples were tested for their inhibition of LPS and PHA induced TNF-α and Il-1α production. Similar to data obtained from experiment 19, these preparations failed to inhibit these mitogen induced cytokine productions.

Conclusions

This is the second time that preparation of Nephronin has resulted in samples that are not effective in inhibiting mitogen induced cytokine production. The only modification of importance, was performing the reaction in air. This is very interesting, considering that when the tubes were being capped under nitrogen, it is certain that under the conditions used, it would not have been possible to remove all of the oxygen, if oxygen is indeed responsible for this phenomenon. Once again the data from this experiment serves as a control. Although in previous experiments appropriate controls have been used to demonstrate the specificity of preparations of Nephronin, these unsuccessful preparations are ideal as a control since they have been taken through the synthesis procedure including the use of POCl3.
Whilst pH adjustment of samples in previous successful synthesis had resulted in the formation of the white fume, this can not be taken as an indication of a successful synthesis, since the same phenomena was observed with these unsuccessful synthesis.

Experiment 21

At this point Hexanoic and Octanoic acid were also received. These two acids will also be used in attempts to synthesis biologically active Nephronin.
The data from experiments 19 and 20 clearly demonstrated that for a successful synthesis, the POCl3 reaction must be performed under nitrogen. Although, as yet no explanation is available as to why this is the case, the results are apparent. The absence or the presence of oxygen is not thought to play an important part in the synthesis of the hypothesised compound. The possibility exists that the synthesis procedure results in the production of a compound that is totally unrelated to the structure of Nephronin.

Aims

1. To synthesis Nephronin under nitrogen.
2. To increase the time of incubation of Na3Citrate with the appropriate acids to allow the reaction to go to completion. It is thought that the incubation of the acid might result in production of acid anhydrides and sodium salt to the acid. The sodium salt of the acid will be removed when the acid

soluble material is removed and the citrate-acid anhydride is used in the POCl3 reaction mixture. The data from previous experiments indicates that in using this procedure a more potent compound is produced. Consequently, the incubation time of the Na3Citrate with the acids is increased from a few hours to at least 24 hours incubation. Furthermore, to increase the chances of the acid and the Na3Citrate mixing, the mixtures were placed on a roller, ensuring that the solid and the acid (liquid) were being mixed constantly. This is particularly important in the treatment of Na3Citrate with acetic acid. In this particular case, the treated Na3Citrate forms a very hard solid that tends to adhere tightly to the wall of the reaction tube. Thus, to ensure mixing of the content of the tubes the tubes were shaken vigorously.

Method

To establish the parameters for the synthesis reaction the following information was used.
The molar volume of the acids and the POCl3 were calculated from their specific gravity (s.g)

Compound	MW	s.g	Volume
Na3Citrate	294.1		
POCl3	153.3	1.645	93.2
Acetic acid (AA)	60.05	1.049	57.24
Propanoic acid	74.08	0.993	74.6
Butyric acid	88.11	0.959	91.87
Hexanoic acid	116.16	0.927	125.3
Octanoic acid	144.22	0.91	158.5

Based on the information given above and the fact that each citrate molecule has three carboxyl groups, for every 3g of Na3Citrate, an excess of 10 fold of each was used.
In the cases of AA, PA and BA, two reaction tubes were set up for comparison between preparations.

The following reactions were set up;

1. 3g Na3Citrate + 5.7ml AA
2. 3g Na3Citrate + 5.7ml AA
3. 3g Na3Citrate + 7.4ml PA
4. 3g Na3Citrate + 7.4ml PA
5. 3g Na3Citrate + 9.2ml BA
6. 3g Na3Citrate + 9.2ml BA

7. 3g Na3Citrate + 12.5ml HA
8. 3g Na3Citrate + 15.8ml OA

The citrate and the acids were mixed very vigorously. In the case of AA + Na3Citrate, the solution became very viscous and stuck to the bottom of the tube. The tubes were mixed very vigorously until the solid was displaced. The tubes were placed on a hybaid roller for 24 hours.

Interestingly, in the case of AA and PA, a white material had solidified on the sides of the tubes with no liquid visible. In the cases of BA, HA and OA, the acid added was still visible as liquid. In these cases (but not AA and PA) the mixtures were centrifuged and the liquid decanted.

In all cases, the tubes were placed under a fume hood to allow evaporation of excess acid for 30-40min.

The following POCl3 reaction tubes were set up.

1. 1.8ml AA + 10ml Hexane + 1 ml POCl3
2. 1.8ml AA + 10ml Hexane + 1 ml POCl3
3. 2.33ml PA + 10ml Hexane + 1 ml POCl3
4. 2.33ml PA + 10ml Hexane + 1 ml POCl3
5. 2.9ml BA + 10ml Hexane + 1 ml POCl3
6. 2.9ml BA + 10ml Hexane + 1 ml POCl3
7. 3.9ml HA + 10ml Hexane + 1 ml POCl3
8. 4.74ml OA + 10ml Hexane + 1 ml POCl3

The tubes were capped under nitrogen and the contents mixed initially by inverting the tubes and then by placing the tubes on a roller. After 30min incubation at RT, the content of the tubes was added to the relevant treated citrate tubes. Once again the tubes were capped under nitrogen before the content of the tubes was mixed by vigorously shaking the tubes. In the case of AA and PA reaction mixtures, the tubes became warm, suggesting that the reaction was exothermic. The contents of both AA and PA reaction tubes looked like a thick creamy coloured material that remained separate from the solvent phase. In the cases of BA, HA and OA, the crystalline did not change in form, except that they formed larger aggregates.

The reaction tubes were placed on a roller and the reactions were allowed to proceed at RT over night. After approximately 20h incubation, the reaction tubes were freeze-dried. The samples took a long time to dry, particularly in the case of PA. In this instance, a milky coloured solution appeared that did not easily dry. To assist freeze-drying of the sample, 5ml of Hexane was added to this material. Addition of solvent caused precipitation of the white material, giving a clear solution. However, further attempts to freeze-dry the sample resulted in the reappearance of a milky coloured material. This is thought to be

due to rapid evaporation of Hexane, and redissolving of the white material in the liquid. The pH of this milky material was adjusted to neutral with 10% ammonium hydroxide.

5ml of Hexane was added to the lyophilised material from other reaction tubes. In the cases of HA and OA, the lyophilised material seemed quite soluble in the solvent.

Rather than attempting to dissolve the lyophilised material in water first, the samples were dissolved directly in a solution of 10% ammonium hydroxide, added gradually. In order to achieve neutral pH, all samples required approximately 20ml of the ammonium hydroxide solution.

During the pH adjustment of the tubes the following observations were made.

In the cases of BA, HA and OH, a tanned layer was visible on top of the aqueous phase. This layer was very liquid in nature, and when mixed with the lower layer phase exhibited what looked like fatty droplets.

In all cases, upon addition of more ammonium hydroxide solution to the mixtures, these upper layers began to disappear. It was noted that in the case of BA, the pH at which the tan layer disappeared was approximately pH 6.0-6.2. However, in the case of HA, the pH was very near pH 7.0. In the case of OA, as the pH of the sample neared pH 7.0, the material turned into a jelly like mass, that became fluid with further addition of ammonium hydroxide. The pH of all samples was adjusted to pH 7.4, and filter sterilised prior to being tested for their biological activity.

Results

Fig 1 shows the inhibition profile of LPS-induced TNF-α production for all the preparations. Duplicate preparations of AA, BA and PA were being tested to assess variability in the potency of preparation in a given biological system. The results indicate that in general, the potency of the preparations increased with increasing carbon chain of the acid. Interestingly, in the case of OA, addition of 10μl of the product resulted in 100% inhibition of TNF-α. Microscopic examination of the cells clearly showed that this preparation of OA was toxic to the cells, since significant cell death and cell lysis was observed. The toxicity of the OA preparation was also observed for PHA stimulation of cells.

The inhibition of PHA induced TNF-α production was as expected.

Conclusions

1. The most important conclusion from this experiment is that biologically active Nephronin can be prepared when the POCl3 reaction is performed under nitrogen.
2. Nephronin can be prepared from longer chain acid. However, it seems that longer chain acids produce toxic by product(s) that are not removed by freeze-drying of the samples.

Experiment 22

In experiment 21, the addition of AA and PA to the Na3Citrate followed with mixing and 24h incubation at RT had resulted in the formation of a white solid. The purpose of pre-treating the Na3Citrate was to remove the sodium salt of the acid and formation of citrate-acid anhydrides.

The extent of the reaction obviously depends on how well the reaction proceeds. It was decided to pre-treat Na3Citrate with either AA or PA and to allow the reaction to proceed over night so that the white solid material was formed. The same volume as the starting volume of each acid is then added to the appropriate solids. The samples are then mixed and after a relatively short incubation time, the resulting liquid would be removed. The treated citrate would be taken through the reaction procedure and the potency of the products tested.

Aims

1. To test whether further treatment of Na3Citrate first treated with either AA or PA with more of the same acids would result in the production of more potent preparations.
2. To test the effects of a number of possible compounds that may be present in the preparations of Nephronin on inhibition of LPS and PHA induced cytokine production.

Method

The following reaction mixtures were set up.

1. 3g Na3Citrate + 5.7ml AA
2. 3g Na3Citrate + 7.4ml PA

The mixtures were mixed very vigorously at first and then placed on a roller ensuring that the acid and the Na3Citrate crystalline were being mixed. They were then incubated over night at RT. After this period, a white milky coloured thick mixture that adhered to the walls of the tubes was visible. Interestingly, within each tube certain portions looked like as hard chalky solids. To each tube the same volume as the starting volume of the appropriate acid was added to the tubes. The content of the tubes were mixed well until the solid had fully mixed with the acid. This resulted in the formation of a milky coloured thick liquid. The mixtures were placed on a roller and incubated for 6-8h at RT. After this time the milky coloured thick solution had become visibly more viscous. The tubes were then centrifuged at 4800 rpm for 10min. Centrifugation of the mixtures had resulted in the separation of a

white precipitate and a clear liquid. In each case the liquid was decanted and the tubes were placed in a fume hood to allow excess acid to evaporate.

The following POCl3 reactions were set up.

1. 1.8ml AA +10ml Hexane + 1 ml POCl3
2. 2.3ml PA + 10ml Hexane + 1 ml POCl3

The tubes were capped under nitrogen prior to mixing the content. The reaction tubes were then placed on a roller and incubated at RT for 30min. After this time, the contents of the POCl3 reaction tubes were added to the pre-treated citrated tubes. Once again the tubes were capped under nitrogen prior to mixing the content of each tube. Upon mixing the reaction mixtures, the tubes became warm indicating that the reaction was exothermic. In both cases, when the solids were mixed with the POCl3 reaction mixture, they transformed into a liquid that remained as a separate phase to the solvent layer. The reaction mixtures were placed on a roller at RT. After this time, the samples were freeze-dried. 5ml of Hexane were added to the lyophilised material. Upon addition of hexane to this material, the white solid visibly separated from the solvent added. The hexane was evaporated under vacuum (freeze-dried). The resulting material looked like dough and would not dry further.

To the dough like material 10% ammonium hydroxide was added, without attempting to dissolve the samples in water first. Upon addition of ammonium hydroxide, a greenish yellow solution was formed. The tubes became very warm. In the case of AA, the greenish-yellow colour seemed to disappear after a short period leaving a somewhat clear solution. In the case of PA, the coloured transparent solution gave way to a cloudy milky coloured solution. After further addition of ammonium hydroxide, a colourless, slightly cloudy solution was observed. The solutions became more transparent as the pH of the samples was adjusted to near neutral. The pH of both samples was adjusted to pH 7.4. Interestingly, in each case approximately 20ml of 10% ammonium hydroxide were required to adjust the pH of the samples to neutral. Because of the high concentration of salts in each preparation, freeze-drying of the samples was hampered by thawing of the samples in the freeze-drying chamber.

Once the samples were freeze-dried, in each case the lyophilised material was resuspended in water and the pH adjusted to pH 7.4 with ammonium hydroxide. The samples were filter sterilised prior to being tested for their biological activity.

Results

Figs 1 and 2, show the inhibition profile of LPS induced TNF-α and Il-1α production by two preparations of Nephronin. The dose dependent inhibition profiles are similar to profiles from previous experiments where the synthesis was considered successful and the products potent.

It is noteworthy that preparation 2, where propanoic acid was used, the product was slightly more potent than the preparation where acetic acid was used. This follows the general trend obtained through out these studies.

Figs 3 and 4 show the inhibition of PHA-induced TNF-α and IL1-α production. Once again the dose dependent inhibition profiles are similar to those previously obtained. Interestingly, in both LPS and PHA induced IL-1α production, the preparations were more potent in inhibiting IL-α when compared to inhibition of TNF-α.

Conclusions

The observation that treatment of Na3Citrate with either acetic acid or propanoic acid resulted in the formation of a chalky looking solid. The formation of such a solid is very much dependent on the incubation time and the thorough mixing of the samples. Earlier studies had shown that, at least in the case of acetic acid treatment of Na3Citrate, the citrate crystals tended to aggregate and adhere to the bottom of the tube. However, vigorous mixing of the mixture allowed formation of the final product. In the case of propanoic acid treatment of the Na3Citrate, although the Na3Citrate crystals did not seem to aggregate in the same manner, the formation of the white aggregate was achieved. Further addition of the acids to this material resulted in the formation of a milky coloured viscus liquid that upon centrifugation separated into a white solid precipitate and a liquid phase. Interestingly, the pH of this liquid was several pH units higher than the acid itself. This suggests that pre-treatment of the citrate with these acids results in formation of a salt, most likely a sodium salt of the acid that seems to remain in the solution. At this stage, one can only speculate as to the nature of the white solid, however, the likelihood of a citrate-acid anhydride can not be excluded.

Experiment 23

The concept in pre-treating the Na3Citrate with the acid was to remove some of the sodium salts that are formed with the addition of the acids to the Na3Citrate molecule. Furthermore, the citrate-acid anhydrides produced by this treatment would enable formation of the hypothesised compound. However, citrate-acid anhydrides would easily hydrolyse to the appropriate ammonium salt of the acid and ammonium salt of the citrate. Given the fact that ammonium acetate is more easily removed by freeze-drying of samples, than ammonium salts of other acid products, led to the following hypothesis. Pre-treatment of Na3Citrate with acetic acid would result in the production of citrate-acid anhydride. If this preparation was then treated with POCl3 reactions where different acids were utilised, different versions of Nephronin could be synthesised.

Aims

To synthesise different versions of Nephronin from pre-treated Na3citrate with acetic acid, and use of different acids in the POCl3 reaction mixture.

Methods

4 tubes, each containing 3g of Na3Citrate were prepared. To each tube 5.7ml of acetic acid was added and the content of each tube was mixed very vigorously. The tubes were placed on a roller over night at RT.

In each case the liquid had disappeared and a white material was produced. Interestingly, in some cases the white material was hard and chalky in nature, compared to white semi-hard material in other tubes. A further 5.7ml of acetic acid was added to each tube and the content of the tube was mixed vigorously. In all cases a thick milky solution was formed. The tubes were placed on a roller and incubated at RT for a further 3-4 hours. After this time, the tubes were centrifuged at 4800 rpm for 10min at RT. The clear liquid was decanted from each tube and the white solids were placed in a fume hood with open top for 30-40min to allow evaporation of excess acid.

The following POCl3 reaction mixtures were set up;

1. 2.33ml of PA + 10 ml Hexane + 1ml POCl3
2. 2.9ml of BA + 10 ml Hexane + 1ml POCl3
3. 3.94ml of HA + 10 ml Hexane + 1ml POCl3
4. 4.74ml of OA + 10 ml Hexane + 1ml POCl3

The tubes were capped under nitrogen. The tubes were placed on a roller to allow mixing of the contents of each tube. After 30min incubation at RT, the content of each tube was poured into numbered tubes. As soon as POCl3 reaction mixtures were added to the tubes, the tubes were capped under nitrogen. The content of each tube was mixed by vigorously shaking the tubes. In each case, the solid material changed to a milky coloured thick solution that remained separate from the solvent phase. Upon mixing the content of each tube, the tubes became warm to the touch, suggesting that an exothermic reaction was in progress.

Interestingly, in the cases of BA, HA and OA, after the addition of POCl3 reaction mixture to the tubes, the white solid material initially changed to a thick white liquid. However, within a few minutes of the reaction, this white liquid changed to a semi solid (dough like) material. The tubes were placed on a roller over night.

Attempts at freeze-drying the samples failed. It was decided to add 5ml of hexane to each tube and remove the solvent soluble material, rather than freeze-dry the samples. Upon addition of hexane to the tubes, followed by mixing, an opaque solvent layer was formed. The tubes were centrifuged at 48000 rpm for 10min. In the case of propanoic acid, two layers were visible. An upper opaque layer and a tanned lower layer. In each case the upper opaque solvent layers were removed.

In an attempt to clean the preparations, 5ml of hexane was added to each tube. The tubes were mixed vigorously and centrifuged as above. Once again an upper opaque layer was observed. Interestingly, in the case of PA, the volume of the tanned layer had reduced considerably. The solvent washing procedure was repeated three more times, at the end of each time, the solvent layer was decanted. At the end of this procedure the solvent layer was no longer opaque.

The solvent layer from each tube was pooled in appropriately numbered tubes. To test the biological activity of the solvent soluble material in the pooled solvent layer, they were freeze-dried. The lyophilised solvent soluble material was very hydroscopic and quickly absorbed moisture.

The precipitates, after cleaning washing with the solvents had become slightly tanned. To dissolve the material in each tube, and to reduce the final volume of the sample, 20% ammonium hydroxide was used. Upon addition of ammonium hydroxide to each tube, a transparent greenish-yellow solution was formed. The tubes became very hot. This transparent coloured solution changed into a white chalky liquid. Upon addition of more ammonium hydroxide, the chalky coloured liquid changed to a slightly cloudy solution. The pH of the samples was adjusted to pH 7.4 and the samples were freeze-dried.

These samples constantly thawed whilst being freeze-dried, leaving a clear solution with a white precipitate. Without mixing the samples, the contents of the tubes were frozen and they were then freeze-dried. Using this procedure resulted in successful freeze-drying of the samples.

In each case the weight of the lyophilised material was approximately 8g, which is three times the starting weight of Na3Citrate.

These samples were dissolved in water and the pH adjusted to pH 7.4 and the final volume was made up to 40ml.

The lyophilised solvent soluble material was dissolved in a few ml of water. The pH of the samples was measured. These samples were very acidic with an average pH of 1.0. Interestingly, whilst adjusting the pH of these samples, similarly to observations made in experiment 21, two layers were formed, an opaque upper layer and a tanned lower layer. Furthermore, as the pH of these samples was being adjusted to neutral, the lower tanned layer disappeared. In the case of OA, the mixture turned into a jelly like material that turned into liquid when more ammonium hydroxide was added, and the pH adjusted to pH 7.4. The important point that must be taken into consideration is that in experiment 21, these observations were made in preparations of Nephronin without the hexane washing procedure, however, in these cases, the solvent soluble material decanted during the hexane washes exhibited these

properties. It seems that the hexane washing procedure might have introduced a cleaning step in the preparation of Nephronin.

All samples were filter sterilised and tested for their biological activity.

Results.

Figs 1 and 2 show the dose dependent inhibition of LPS-induced TNF-α and IL-1α by four different preparations of Nephronin. In these cases the Na3Citrate was pre-treated with acetic acid. Different acids were then used in the POCl3 reaction and the synthesis procedure.

The inhibition profiles are very similar to inhibition profile from previous experiments, except that in this case, the preparations where acids of longer carbon chains were used, seemed relatively less potent than preparations where acids with shorter carbon chain length was used.

Fig 1 shows the inhibition of LPS-induced TNF-α production. Preparation 4, where Octanoic acid was used in the synthesis is less potent than preparation 3, where Hexanoic acid was used. Preparations 1 and 2, where Propanoic acid and Butyric acids were used exhibited very similar potency.

Fig 2 shows the dose dependent inhibition of LPS-induced IL-1α for the same preparations.

Although these preparations exhibited very high inhibition of IL-1α when only 10μl of each preparation was used, a similar pattern in the inhibition was observed. Preparations 3 and 4 corresponding to Hexanoic and Octanoic acid preparations respectively were less potent than those exhibited by Propanoic and Butyric acid, preparations 1 and 2 respectively.

Figs 3 and 4 show the dose dependent inhibition profile of PHA induced TNF-α and IL-1α for the same preparations. The differences in the potency of these preparations seen for LPS-induced cytokine production are no longer obvious. Once again, these preparations seem more potent in inhibiting IL-1α production than TNF-α. However, they remain very potent in inhibiting these cytokines and the synthesis of Nephronin is considered successful.

Conclusions

Pre-treatment of Na3Citrate with acetic acid followed by the synthesis of Nephronin with different acids used in the POCl3 reaction, resulted in preparations that are potent in inhibiting TNF-α and IL-1α. However, contrary to previous observations, in these instances, where acids of longer chain length was used, the resulting preparations were less potent. It must be born in mind that the observed in the potency of these preparations is relatively small, however, the differences in their potency remain significantly different from each other.

Experiment 24

Na3Citrate is used for the synthesis of Nephronin. However, Na3Citrate can itself produce some false results. This may be due to the chelating effects of Na3Citrate. In order to test for the chelating effect of Na3Citrate in the preparations of Nephronin, different amounts of CaCl2 were added to the two preparations of Nephronin from experiment 23. Preparations 1 and 4 were used to represent both sides of the spectrum, for the carbon chain length of the acids used in the synthesis.

Aims

1. To test the effect of added CaCl2 to preparations of Nephronin and control ammonium citrate buffer for their ability to inhibit LPS and PHA induced cytokine production.
2. Inhibition of commercial streptococcal toxins, Spe A, Spe B induced cytokine production by 0.25M ammonium citrate and preparations of Nephronin with and without CaCl2.

Method

The concentration of citrate in the preparations of Nephronin was calculated on the basis of the starting amount of Na3Citrate used in the synthesis and the final volume of the product.

In experiment 23, Nephronin was prepared using 3g of Na3Citrate. The final volume of the product was 40ml. Taking into account that Na3Citrate has a molecular weight of 294, 3g of Na3Citrate in 40ml would give a concentration of 0.25M.

To prepare the control for this experiment, 0.25M Citric acid was prepared;

Citric acid has a molecular weight of 210.

1.05g of citric acid in a final volume of 20ml, gives a concentration of 0.25M. However, the citric was first dissolved in 10ml of water. The pH was adjusted to pH 7.3 with ammonium hydroxide and the final volume was then made up to 20ml.

To test the effect of CaCl2, a solution of 2.5M CaCl2 was prepared.

CaCl2.H2O MW 147

3.67g dissolved in 10ml gives a final concentration of 2.5M.

On the basis of the Na3Citrate concentration used in the synthesis procedure, different ratios of the CaCl2 was added to preparations of Nephronin or the ammonium Citrate control buffers.

The following tubes were set up as below.

Preparations 1and 4 from expt 23, where propanoic acid and Octanoic acid were used respectively. In each case 0.5ml aliquot of the samples were used in this study.

1. 25µl of 2.5M CaCl2 was added to 0.5ml aliquot of prep 1. 2M:1M, Nephronin to CaCl2
2. 50µl of 2.5M CaCl2 was added to 0.5ml aliquot of prep 1. 1M:1M, Nephronin to CaCl2
3. 75µl of 2.5M CaCl2 was added to 0.5ml aliquot of prep 1. 1M:1.5M, Nephronin to CaCl2
4. 25µl of 2.5M CaCl2 was added to 0.5ml aliquot of prep 4. 2M:1M, Nephronin to CaCl2
5. 50µl of 2.5M CaCl2 was added to 0.5ml aliquot of prep 4. 1M:1M, Nephronin to CaCl2
6. 75µl of 2.5M CaCl2 was added to 0.5ml aliquot of prep 4. 1M:1.5M, Nephronin to CaCl2

For controls 0.25M ammonium citrate, pH 7.3 was used.

7. 25µl of 2.5M CaCl2 was added to 0.5ml aliquot of 0.25M-ammonium citrate, pH 7.3. 2M:1M, Citrate to CaCl2
8. 50µl of 2.5M CaCl2 was added to 0.5ml aliquot of 0.25M-ammonium citrate, pH 7.3. 1M:1M, Citrate to CaCl2
9. 75µl of 2.5M CaCl2 was added to 0.5ml aliquot of 0.25M-ammonium citrate, pH 7.3. 1M:1.5M, Citrate to CaCl2
10. No addition was made. 0.25M ammonium Citrate, pH 7.3.

Upon addition of samples to the tubes containing the appropriate amount of CaCl2, a white precipitate was formed. However, the same did not happen when ammonium citrate was added to the tubes containing CaCl2. Precipitates were visible after freeze-thawing the samples. This suggests that in the case of ammonium citrate, for the calcium citrate precipitate to form, a longer time is required.
In an attempt to identify which of the constituents of the preparations of Nephronin were likely to form precipitate in the presence of CaCl2, buffers used as controls, such as ammonium phosphate, were mixed in the same proportions with the solution of 0.25M ammonium citrate. The tests showed that ammonium citrate formed similar precipitate as those observed with preparations of Nephronin, with ammonium phosphate. Considering the fact that preparations of Nephronin contain some inorganic phosphate, formation of calcium phosphate precipitate is not surprising. The sample mixtures were centrifuged in a microcentrifuge and the supernatant was decanted for testing.
When evaluating the data, the presence of CaCl2 in the solution must be taken into consideration.

The RPMI 1640 being used does not seem to contain CaCl2. However, other media such as Minimum Essential Medium Eagle contain 264mg of CaCl2/litre of media. Since CaCl2.H2O has a MW of 147, the concentration of CaCl2 in the media is approx 2mM.

After the addition of Nephronin to the RPMI with cells, the final concentration of CaCl2 in the media would be in the range of 1-4mM.

Results

Figs 1-4 show the dose dependent inhibition of LPS-induced TNF-α and IL-1α at 24 and 48h post-stimulation by preparation 1 from experiment 23, where propanoic acid was used in the synthesis procedure, with and without the addition of CaCl2. The data clearly shows that addition of CaCl2 to preparation 1 from experiment 23 does not appear to have any effect on the inhibition of LPS-induced TNF-α and IL-1α production. These data confirm earlier results that Nephronin binds to the LPS molecule and prevents it from stimulating the cells to produce these cytokines. Furthermore, removal of precipitates formed with the addition of CaCl2 seems to enhance the observed inhibition.
Figs 5-8 show the dose dependent inhibition of LPS-induced cytokine production at 24 and 48h post-stimulation by preparation 4 from experiment 23, where Octanoic acid was used in the synthesis procedure, with and without the addition of CaCl2. Once again the addition CaCl2 to this preparation does not appear to have any effect on its ability to inhibit LPS-induced cytokine production.

Figs 9-12 show the effect of 0.25M-ammonium citrate with and without the addition of CaCl2 on the LPS-induced TNF-α and IL-1α production. Ammonium citrate seems to have a slight inhibition effect on the production of TNF-α at 24h post-stimulation, particularly when higher amounts of ammonium citrate were used. However, this inhibition is no longer observed by 48h post-stimulation (Figs 9 and 11). Interestingly, ammonium citrate, with and without the addition of CaCl2 show a good dose dependent inhibition of LPS-induced IL-1α. This may be due to the fact that production of IL-1α is more sensitive to ammonium citrate and any products that are formed after the addition of CaCl2 (Figs 10 and 12).

Figs 13-16 show the dose dependent inhibition of PHA induced TNF-α and IL-1α production by preparation 1 from experiment 23.
Figs 13 and 14 show the inhibition of PHA induced TNF-α and IL-1α, 24h post-stimulation respectively. Although the inhibition profile for TNF-α does not seem to alter with the addition of increasing amounts of CaCl2, when only 10μl of samples were used, a difference in the amount of inhibition of PHA-induced IL-1α is observed. However, at 30μl of samples this difference is abolished.
Fig 15 shows the inhibition profile for TNF-α 48h post-stimulation. Interestingly, when 10μl of samples were used, a difference in the amount of inhibition for TNF-α was seen. This difference is abolished when 30μl of the samples were used.

Fig 16 shows the inhibition profile for IL-1α 48h post-stimulation. The difference in the amount of inhibition observed at 24h post-stimulation had became considerably greater, particularly when 1.5M ratio of CaCl2 was used to 1M of the Na3Citrate in the preparation of Nephronin. Once again this difference is totally abolished when 30μl of samples were used.

Figs 17-20 show the dose dependent inhibition profile for PHA induced cytokine production by preparation 4 from experiment 23. The results are very similar to those obtained for preparation 1, except that preparation 4, where Octanoic acid was in the synthesis of Nephronin seems more susceptible to treatment with CaCl2 and the differences observed, with the addition of increasing amounts of CaCl2 seem to be more pronounced.

Figs 17 and 18 show the dose dependent inhibition of PHA-induced TNF-α and IL-1α, 24h post-stimulation. Fig 17 shows that irrespective of the amount of CaCl2 added to Nephronin, preparation 4 the samples remained very potent in inhibiting TNF-α production. However, the inhibition profile shown in Fig 18, for IL-1α for the same samples show significant reduction in the potency of the sample with increasing amounts of CaCl2.

Figs 19 and 20 show the inhibition profile for the samples 48h post-stimulation by PHA. The decrease in the potency of these samples for inhibiting PHA-induced cytokine production is reduced considerably, particularly in the case of IL-1α.

Figs 21-24 show the effect of a solution of 0.25M ammonium citrate pH 7.4, with and without increasing the amount of CaCl2.

Fig 21 shows that although 0.25M ammonium citrate seem to only inhibit PHA-induced TNF-α production by 33%, none of the samples containing CaCl2 showed any inhibition of TNF-α. Interestingly, ammonium citrate with CaCl2 seemed to increase the levels of TNF-α production. Fig 22 shows the effect of the same samples on the PHA-induced IL-1α production. Once again, untreated ammonium citrate could inhibit PHA-induced IL-1α production by 80%. However, this effect was significantly reduced when CaCl2 was added to ammonium citrate.

Figs 23 and 24 show the effect of the same samples on PHA-induced TNF-α and IL-1α 48 post-stimulation. Although the result obtained 48h post-stimulation is similar to the earlier results, the effects seen by ammonium citrate and ammonium citrate with CaCl2 is considerably reduced.

The data presented here clearly shows that the presence of citrate accounts for some of the effect that Nephronin displayed. However, preparations of Nephronin are considerably more potent in their effects. Furthermore, given the fact that Nephronin is a modification of the citrate molecule, it is not unexpected that citrate itself might show some non-specific activities.

Figs 25-32 show the effects of preparations 1, 4 and ammonium citrate with and without the addition of CaCl2, in a ratio of 1M:1M on the basis of the citrate in preparations of Nephronin and ammonium citrate solution, on streptococcal toxins Spe A and Spe B induced TNF-α and IL-1α production.

Fig 25 shows the effect of these samples for TNF-α production, 24h post-stimulation when 10μl of each sample was used. Preparations 1, 4 and citrate seem to effectively inhibit TNF-α production, although preparations 1 and 4 are considerably more potent. However, the amount of inhibition observed for these samples are significantly reduced with the addition of CaCl2. Interestingly, citrate alone seems to have very little effect on the Spe B induced TNF-α production.

Fig 26 shows the effect of the same samples 48h post-stimulation. It is noteworthy, that the amount of inhibition observed for the untreated preparations 1 and 4 are reduced and, where CaCl2 was added to these preparations, very little if any inhibition was observed. Once again, Citrate alone does not seem to inhibit Spe B induced TNF-α.

Fig 27 shows the effect of the samples 24h post-stimulation, except that 30μl of each sample was used. Once again, although Citrate shows significant amounts of inhibition, preparations of Nephronin are considerably more potent. Interestingly, at this concentration, citrate alone effectively inhibits Spe B induced TNF-α production.

Fig 28 shows the effect of 30μl of each sample on TNF-α production, 48h post-stimulation. Once again, although citrate seems to significantly inhibit TNF-α production, preparations of Nephronin with and without CaCl2 are more potent.

Fig 29 shows the effect of the same samples, when 10μl of each was used, on Spe A and Spe B induced IL-1α production, 24h post-stimulation. In the case of Spe A, citrate with and without CaCl2 seems to inhibit IL-1α production considerably more than both preparations of Nephronin. However, in the case of Spe B, both preparations of Nephronin without CaCl2 inhibit IL-1α considerably more than citrate alone. Interestingly, preparation 4 with the addition of CaCl2 shows similar inhibition to that of Citrate with and without added CaCl2.

Fig 30 shows the effect of 10μl of these samples 48h post-stimulation. Citrate with and without CaCl2 seems to inhibit Spe A induced IL-1α production similarly to both preparations of Nephronin with and without CaCl2. Whereas in the case of Spe B, both preparations of Nephronin effectively inhibit IL-1α production, this inhibition is greatly reduced with the addition CaCl2. However, ammonium citrate inhibition of Spe B induced IL-1α does not seem to change significantly in the presence of CaCl2.

Figs 31 and 32 show the effect of 30μl of the same samples at 24 and 48h post-stimulation on Spe A and Spe B induced IL-1α production. All samples with and without CaCl2 effectively inhibited IL-1α production, although the general trend remains the same, in that with the addition of CaCl2, the potency of these samples is reduced.

Conclusions

There are several conclusions that can be drawn from these studies,

1. Addition of CaCl2 to preparations 1 and 4 of Nephronin did not interfere with their ability to inhibit LPS-induced TNF-α and IL-1α production. These data support the earlier findings that Nephronin inhibits LPS by binding to the lipid A moeity and neutralising the toxic effects of LPS.
2. Both preparations of Nephronin effectively inhibit PHA-induced TNF-α. However, the fact remains that after the addition of CaCl2, the amount of inhibition, particularly in the case of IL-1α is significantly reduced. This might suggests that in the case of PHA, inhibition of cytokine production by Nephronin is achieved by inhibition of cytokine production rather than by binding to the PHA molecule and inhibiting its biological effects.
   Given the fat that Nephronin is a modification of Citrate, it is not surprising that citrate displays some of the non-specific activities ascribed to Nephronin.
3. Although both preparations of Nephronin can effectively inhibit Spe A and Spe B induced TNF-α and IL-1α production, the fact that citrate alone also seem to show similar ability, suggests that the observed inhibition is non-specific. Furthermore, the decrease in the observed inhibition with the addition of CaCl2 suggests that a considerable amount of the inhibition is due to the citrate rather than Nephronin.

It must be born in mind that these preparations of Nephronin comprise of citrate molecule and as a consequence, Nephronin itself would display such activities. Further experiments are required to investigate the importance these non-specific effects.

Effects of possible compounds that may be present in the preparations of Nephronin on LPS and PHA induced cytokine production.

In order to test whether the inhibition of mitogen induced cytokine production is specifically due to preparations of Nephronin. The toxic effects of some components that are thought to be present in these preparations were tested for their effect on LPS induced TNF-α and IL-1α production.
The following were prepared and tested

1. 0.1M ammonium acetate, pH 7.4

2. Ammonium Citrate. To determine the effect of ammonium citrate in a way that would correspond to the concentration present in the preparations, 0.3g citric acid was weighed and dissolved in water. The pH of the sample was adjusted to pH 7.4 with 10% ammonium hydroxide. The final volume of the sample was adjusted to 5ml.
3. Use of POCl3 in the synthesis procedure, might lead to the formation of ammonium phosphate after pH adjustment. Given the fact that 100µl of POCl3 is used in the synthesis procedure, 100µl of orthophosphoric acid was used for this purpose. To this 3ml of water was added and the pH adjusted to pH 7.4 with 10% ammonium hydroxide.

1 ml of the liquid phase after treatment of Na3Citrate with AA or PA was also used in these studies. The pH of these samples was adjusted to pH 7.4 with ammonium hydroxide and the final volume made up to 5ml with water. The samples were filter sterilised and their effect was investigated with the samples in experiment 22.

Results.

In each case 40µl of each sample was added to cells in a final volume of 1ml. 0.1 ammonium acetate pH 7.4 did not have any inhibitory effect on the LPS induced cytokine production.

40µl of ammonium citrate showed 40% inhibition of TNF-α and up to 80% inhibition of IL-1α. Whereas, preparations of Nephronin at these concentrations show 90-100% inhibition of LPS induced TNF-α production. Almost 100% inhibition of IL-1α production was observed when only 10µl of these preparations were used. The fact that such a preparation of ammonium citrate at pH 7.4 can exhibit such an effect may be due to high salt concentration and non-specific binding effects of citrate.

Ammonium phosphate did not show any inhibitory effect.

Supernatant from acid treatment of citrate, at pH 7.4 was very toxic to the cells. Microscopic observation of the cells showed high cell death and cell lysis.

Conclusions

The effect of control samples on the inhibition of LPS-induced cytokine production shows that,

1. 40µl of ammonium acetate at pH 7.4 does not effect the assay system.
2. 40µl of ammonium phosphate at pH 7.4 does not effect the assay system.
3. 40µl of ammonium citrate causes inhibition of LPS-induced TNF-α and IL-1α production. This is thought to be due to the toxic effects that this compound may have on the cells. Microscopic

examination of the cells indicated that when ammonium citrate was added to the PBMC, stimulated with 1ng/ml LPS, showed some cell death.

4. Addition of acid supernatant from treatment of Na3Citrate to PBMC caused high cell death. Centrifugation of the cell suspension after 24h post stimulation and at 2000 rpm did not show any cell pellets, suggesting that the majority of the cells were lysed. Consequently, the observed inhibition effect is due to cell death rather than inhibition of cytokine production.

These data indicate that preparations of Nephronin are effective in inhibiting mitogen induced cytokine production. However, purification steps are required to remove some of the contaminating compounds that may be produced during the synthesis procedure.

Specific interaction of Nephronin with molecules on the surface of cells.

The ability of Nephronin to specifically interact with cell surface carbohydrates indicated that Nephronin may have other biological activities. To test for these activities, a number of experiments were initiated. However, these experiments, and the results do not cover the full spectrum of activities that may be ascribed to Nephronin.

Effect of Nephronin on normal and tumourogenic cell lines.

Previous observations had shown that treatment of confluent human umbilical vein endothelial cells, with isolated Nephronin, had resulted in the separation of the cells from the substratum. However, trypan blue staining of the cells for viability had shown that these cells remained viable, at least for the duration of the experiment.

To test the effect of synthesised Nephronin on other cell types, normal mouse muscle cell line (C2C12) and mouse neuron tumourogenic cell (B35) lines were used.

Methods

The cells were grown to confluence in 24 well culture plates in DMEM media supplemented with 10% FCS and penicillin/streptomycin antibiotics.

Prior to the addition of Nephronin, the appearance of the cells was observed microscopically. In the case of C2C12 cell line, the cells had a characteristic dendretic appearance, with long protruding tentacles. The B35 cells of the neuron linage showed a characteristic appearance of nerve cells with long tentacles associated with such cells.

Results

Increasing volumes of 10, 20, 30 and 40µl of the four preparations of Nephronin from experiment 23 were added to these cells, to a final volume of 1ml. After the addition of Nephronin to the cells, the appearance of the cells was monitored constantly under a microscope.

Immediately after the addition of Nephronin, they changed shape to round spherical cells without the tentacles, in both cases, irrespective of the volume of Nephronin added to the cells. Interestingly, the surface of the cells seemed to have a frilled look about them. However, they remained bound to the culture plates, within the first 2h of the study. After this time, the C2C12 cells were found to have separated from the substratum and formed large cell clumps. Although, similar cell clumps were also observed with the B35 cells, appearance of blebs on the cells suggested that Nephronin was toxic to B35 cells.

The cells were incubated in media containing Nephronin for 5 days, whilst regular observations were made. After 24h incubation of the cells at 37°C, a large number of the C2C12 cells were found as cell suspension in clumps. This effect was very much dependent on the dose of Nephronin used. Where 10µl of Nephronin was used, the number of clumps was considerably less. Furthermore, a number of cells had begun to adhere to the plate and demonstrated their characteristic appearance. Interestingly, this effect was more pronounced with preparations of Nephronin where acids of longer carbon chains were used. For example, cells with preparation of Nephronin using octanoic acid exhibited a more normal appearance than cells treated with preparation of Nephronin using propanoic acid.

Incubation of B35 cells with these preparations of Nephronin, after 24h resulted in the appearance of small vesicles, which was ascribed to cell lysis. This effect was much more pronounced with larger volumes of Nephronin. However, after 48h incubation of the cells, this dose dependent lysis could not be distinguished. Almost total cell death was observed after 96h incubation of B35 cells with any one of the four preparations of Nephronin.

In both cases, after 5 days, the cell suspensions were centrifuged at 1300 rpm for 7min. The cells were washed once in PBS and then resuspended in DMEM media. The cells were added back to the appropriate wells in the culture plates and incubated at 37°C/5% CO2. Where 10 and 20µl of preparations of Nephronin were used, a significant number of cells had remained bound to the plate. However, where the cells had separated from the substratum, after less than 2h incubation, they adhered back to the plastic. It is noteworthy, that these cells retained their spherical shape, even after 48h incubation. Trypan blue staining of the C2C12 cells suggested that these cells were viable.

In the case of B35 cells, some spherical cells could be seen that did not take up trypan blue, however, close examination of these cells showed membrane blebing, suggesting the onset of apoptosis.

Conclusions

The observed effects of four preparations of Nephronin on these two cell lines, confirms interaction of Nephronin with cell surface molecules. Given the fact that preparations of Nephronin were toxic to B35 but not C2C12 suggests specific interaction of Nephronin with cell surface molecules. However, the fact that these cells did not regain their original shape, particularly at higher concentrations of these preparations, might suggest that

1. These preparations of Nephronin are also toxic to C2C12 cells but to a lesser degree
2. The cell surface differences between a normal and a tumourogenic cell line, enables specific targeting of tumour cells by Nephronin.

Further studies are required to demonstrate the effect of Nephronin with a large number of cell lines, in particular cells of the same lineage. Furthermore, comprehensive experiments are required to demonstrate the onset of cell death/apoptosis, rather than microscopic observation.

Effect of Nephronin on bacterial adhesion to epithelial cell.

Introduction

Enteropathogenic *E.coli* (EPEC) carry virulence factors in the form of adhesins including plasmid-encoded fimbriae and a chromosomally borne adhesin. These adherent *E.coli* alter epithelial cell morphology and the cytoskeleton of host cells (in this model a T84 epithelial cell line) as a mechanism to allow effective attachment and thus colonisation. Infection of confluent T84 cells with EPEC usually results in bacterial adherence (as measured by a bacterial binding assay).

Aims

1. To investigate the effect of Nephronin on bacterial binding to epithelia cells.
2. To test whether Nephronin has any bactericidal activity, viability of bacteria, after addition of Nephronin is also to be test.

Methods:

Inhibition of bacterial binding to epithelial cells.

Immortalised human colonic epithelial cells (T84 cells) were grown to confluence in culture plates at 37°C with 5% CO2 in DMEM/F12 medium supplemented with 10% foetal bovine serum (FBS) and penicillin/streptomycin antibiotics.

Once the cells had reached confluence, the media was replaced with an antibiotic-free media, 40µl/ml of Nephronin was added to the cells. The cells were then inoculated with 10^7 colony forming units (CFU) of EPEC. Preliminary experiments had shown that addition of Nephronin to the inoculated cells, reduced the extent to which the media had become acidic, compared to inoculated cells that were not treated with Nephronin, indicated by the change in colour of the media. However after approximately 3h incubation, the colour of the media had begun to change to yellow indicating that the media was becoming acidic.

Consequently, after a three hour incubation of the treated epithelial cells with the inoculum, a second dose of Nephronin was added to the cells and incubated for a further three hours. At the end of the 6h infection period, cells were washed 5 times with PBS to remove any non-adherent bacteria. 5ml of sterile distilled water containing 0.1% BSA was added to cells, and incubated at 4°C for 30min. The cells were then lysed by vigorous pipetting and 10-fold serial dilutions of the cell lysate suspension were plated on LB agar plates, and were incubated at 37°C overnight. The following day, bacterial CFU were counted at the dilutions that yielded between 25 – 250 CFU, as this is the optimal result, and bacterial binding to T84 monolayers was assessed by normalising the CFU/ml in treated cell lysate to control CFU/ml (expressed as a percentage).

Effect on bacterial viability/growth

To test whether Nephronin had any bactericidal activity, the rate of EPEC growth was measured in broth with and without Nephronin. To 10 mls of Brain Heart Infusion (BHI) broth, 500µL Nephronin was added. For control, the same media without Nephronin was used. In each case, the media was inoculated with 10^3 CFU EPEC. The tubes containing the inoculated broth were placed in a 37°C shaking water bath. After a 2h incubation, a further 500µl of Nephronin was added to the tube treated with Nephronin and incubated for a further 2h. At the end of this period, 10-fold dilutions were performed on the inoculum and then plated onto LB agar plates and were incubated overnight at 37°C. The following day, bacterial CFU were counted as previously described and bacterial viability was expressed as CFU/ml.

Results:

Bacterial binding assay

Treatment with Nephronin both at the beginning of the inoculation period and then again 3 hours post-inoculation, resulted in a significant reduction in bacterial binding to T84 cells. Bacterial binding in

controls was at a concentration of 9.4 x 10^7 CFU/ml, whereas in the treated sample, this was reduced to 4.1x10^7 CFU/ml. This represents a 60% reduction in bacterial binding.

A change in pH, as indicated by a change in the colour of the media, reflects the extent of infection of the cells by the bacteria. The colour of the media of the treated cells did not change colour as rapidly as the control cells. Interestingly, even at the conclusion of the experiment, the colour of the media had not changed as dramatically, compared to that of the control cells. Interestingly, addition of Nephronin to the epithelial cells did not result in the separation of cells from the substratum.

Viability assay

Treatment with Nephronin had no effect on the viability of the bacteria over a 4 hour growth period. The viability count in the untreated sample was 7.5x10^5 CFU/ml and in the Nephronin-treated sample was 1.6x10^6 CFU/ml. This is a significant difference ($p < 0.0001$) which demonstrates that Nephronin is not toxic to bacteria.

Conclusions

The ability of Nephronin to inhibit EPEC bacterial adhesion to epithelial cells, without demonstrating any bactericidal activity, indicates that Nephronin specifically interacts with the cell surface of these cells, thus inhibiting bacterial adhesion. However, the fact that addition of Nephronin to the cells did not result in the separation of cells from the substratum, suggests that Nephronin interacts differently with different cell types. In this instance, inhibition of bacterial adhesion to epithelial cells, results in reduced bacterial colonisation.

Inhibition of Herpes Simplex Virus (HSV) infection by Nephronin

Heparan sulphate serves as a receptor for adherence of herpes simplex viruses, chlamydia trachomatis, Neisseria gonorrhoeae, and, indirectly human immunodeficiency virus (Herold et al. 1997). HSV causes many disease states, including mucosal lesions, encephalitis or disseminated infection in immunocompromised host. These diverse clinical manifestations reflect the capacity of the virus to infect both epithelial and neuronal cell types (Immergluck et al. 1998). Previous studies have shown that the first step in herpes virus infection is attachment to heparan sulphate molecule on the cell surface of cells (Laquerre et al. 1998; Dyer et al. 1997; Williams and Straus, 1997). Given the fact that Nephronin specifically binds to heparin and heparan sulphate as well as other cellular membrane components, suggests that Nephronin may be a prime candidate for inhibiting HSV infection.

Aims

Since Nephronin specifically binds to heparan sulphate, its ability to inhibit herpes virus infectivity of keratinocytes is to be tested,

1. Pre-incubation of keratinocytes with Nephronin 24h prior to addition of HSV inoculum.
2. Simultaneous addition of Nephronin and the HSV inoculum.

Methods

Cultures of Human keratinocytes were prepared as previous described (Mikloska et al. 1996). Preparations of Nephronin from experiment 23 were used in this experiment to test the effect of Nephronin on the inhibition of herpes virus infectivity.
Identical keratinocyte culture plates were used, where in one instance up to 50µl of each preparation was added to the cells and incubated for 24h prior to inoculation with the virus. Alternatively, preparations of Nephronin were added simultaneously as the virus inoculum.
Plaque reduction assay was performed to ascertain HSV infectivity. More comprehensive experiments are also being carried

Results

Where Nephronin was added to the keratinocyte cultures 24h prior to the addition of HSV inoculum, 25-30% inhibition of HSV infection was observed. However, where Nephronin was added simultaneously as the HSV inoculum 100% inhibition of HSV was observed.
It is noteworthy that these cells did not separate from the substratum when Nephronin was added to the cell cultures.

Conclusions

Addition of Nephronin with the HSV, resulted in 100% inhibition of HSV infectivity. Given the fact Nephronin binds to heparan sulphate suggests that addition of Nephronin blocks the heparan sulphate binding sites for HSV binding, thus inhibiting infection. Alternatively, since Nephronin binds to heparan sulphate, might enable Nephronin to bind directly to the virus and inhibiting its infectivity. This is more plausible possibility since addition of Nephronin 24h prior to the addition of HSV inoculum inhibited infectivity by only 25-30%.
Where Nephronin was added to the cells 24h prior to inoculation, suggests that Nephronin might be taken up by the cells and degraded, and the heparan sulphate on the cell surface regenerated. This

observation supports that concept that Nephronin might bind directly to the virus, since the plaque reduction assay takes longer than 24h.

Inhibition of HIV virus infection of macrophages.

HIV virus binds to the cell surface of cells by a virus glycoprotein GP120. The binding of GP120 to the cell surface receptors, CCR5 and to lesser extent CCR3 and CCR2, is the first step in HIV infection of blood or tissue derived macrophages (Naif et al. 1998). However, there is evidence that heparan sulphate as well as other cell surface sugar moieties indirectly play a role in HIV infectivity.

On the basis that Nephronin binds to heparin and heparan sulphate as well other sugar moieties, suggested that Nephronin might be able to inhibit HIV virus infection of macrophages. Alternatively, since Nephronin binds to a host of sugar moieties, it might also be able to bind directly to glycoproteins, such as GP120 and inhibit infectivity of cells by HIV virus.

Aims

To test the effect of Nephronin on the inhibition of HIV infectivity,

1. Nephronin was added to macrophages and incubate for 24h prior to the addition of HIV inoculum.
2. Nephronin was added to the cells at the same time as the HIV inoculum.

Methods

Adherent macrophages were prepared from human blood. Preparations 7, 8 and 9 from experiment 17 were used in these studies. Up to 40µl of each preparation was used in these studies. Preparations of Nephronin were either added to the cells 24h prior to addition of HIV inoculum, or added simultaneously as the HIV inoculum. The cells incubated with the HIV inoculum for 4h. After this time, the cells were washed three times with PBS and finally placed in the media. The susceptibility of macrophages to HIV infection was measured by the concentration of extracellular p12 antigen and levels of intracellular HIV DNA by quantitative PCR.

When Nephronin was added to the macrophages for pre-incubation of the cells with Nephronin caused the cells to separate from the substratum. It was thought that Nephronin was toxic to the macrophages. However, trypan blue staining of the cells for cell viability showed over 95% of the cells were viable. It was decided to proceed with the experiment. After 24h incubation of macrophages with preparations of Nephronin, the cell suspension were centrifuged, washed with PBS and the resuspended cells were added back to the chambers of the tissue culture plates. The macrophages very quickly adhered to the plate. After this time, the HIV inoculum was added to the pre-treated.

Where Nephronin was added simultaneously as the HIV inoculum, very few of the macrophages separated from the substratum. Nevertheless, 4h after the addition of the inoculum, the culture supernatants were centrifuged and the macrophages that had separated from the plate were added back to the appropriate chambers.

Results

Addition of Nephronin to the macrophages 24h prior to the addition of HIV inoculum did not inhibit HIV infectivity. However, when Nephronin was added simultaneously as the inoculum, approximately 30% inhibition was observed.

A more comprehensive set of experiments are currently under way to demonstrate inhibition of HIV infection by Nephronin in macrophages and monocytes.

Animal Studies.

Toxicity of Nephronin.

Up to 100µl of preparations of Nephronin from experiment 23 were used in these studies. Mice were injected either;

1. Subcutaneously
2. Intraperitoneal
3. Intramuscular

There was no visible toxicity that could be associated with the injection of any one of the preparations of Nephronin.

Comprehensive studies are being carried out to demonstrate the endotoxin neutralising ability of Nephronin.

General Discussion

Nephronin is a low molecular weight, non-proteinaceous compound that was originally isolated from the urine of children with steroid responsive nephrotic syndrome. This compound was shown to specifically bind to heparin and heparan sulphate. The fact that Nephronin does not bind to other glycosaminoglycans, such as chondroitin sulphate and dermatan sulphate, suggests that binding of Nephronin to heparin and heparan sulphate is site directed rather than binding on the basis of charge.

This binding was further confirmed by histochemical studies demonstrating that Nephronin blocked staining for heparan sulphate on the surface of endothelial cells. The fact that Nephronin caused the separation of endothelial cells from the substratum without any apparent damage to the cells is of particular interest.

The tests for the endotoxin neutralising ability of Nephronin was initiated, based on the observation that;

1. Nephronin is a negatively charged molecule with specificity for other negatively charged molecules such as heparin, and
2. its co-purification with lipids (affinity for lipids), at least in the early stages of purification

suggested that Nephronin might behave as a cationic detergent. Consequent studies have shown that Nephronin potently inhibits endotoxin in the LAL assay system. Furthermore, this compound was also shown to agglutinate gram negative bacteria but not gram positive bacteria.

The ability of Nephronin to bind to heparin and heparan sulphate suggested that Nephronin might interfere with blood coagulation. The phenomena that Nephronin did not interfere with the anti-coagulation ability of heparin, but itself could inhibit coagulation in the same manner as heparin is very important when considering Nephronin as a therapeutic compound in the treatment of sepsis.

The hypothesis, which led to the successful synthesis of Nephronin, has opened a new dimension in synthesising a range of compounds for use as therapeutic drugs.
The synthesised Nephronin has been shown to have many of the activities ascribed to the isolated Nephronin.

The list below shows some of the biological activities ascribed to Nephronin.

1. Synthesised Nephronin neutralises endotoxin-induced cytokine production.
2. The synthesised Nephronin binds to Heparin and inhibits its migration on cellulose acetate electrophoresis.
3. The synthesised Nephronin inhibits Concanavalin A and PHA stimulation of human lymphocytes.
4. The synthesised Nephronin inhibits stimulation of lymphocytes by streptococcal membrane protein (M-protein), and streptococcal toxins (superantigens) Spe A and Spe B.
5. Nephronin inhibits adhesion of pathogenic E.coli to a gut cell line and precents bacterial invasion of cells.
6. Inhibition of herpes simplex virus infection
7. HIV infectivity

1. The synthesised Nephronin neutralises endotoxin-induced cytokine production.

To test the bioactivity of the synthesised Nephronin, human lymphocytes were isolated from buffy coat (concentrated White Blood Cell preparation, supplied by the Blood Bank). Lymphocytes were stimulated with endotoxin (*E.coli*, serotype 055:B5) in the presence and absence of the synthesised compound. For positive controls, a number of biological stimulators were used, such as PHA, Concanavalin A, streptococcal bacterial membrane protein (M protein) and enterotoxins (superantigens).

Sandwich ELISA for the measurement of the cytokines produced post-stimulation of the cells was established. Sandwich ELISA for TNF-α, and IL-1α was routinely used for the measurement of these cytokines in the culture supernatant of the activated cells post-stimulation. Cells were harvested at 24, 48 and 72 hours post-stimulation and the culture supernatants used for the determination of the cytokine levels.

Fig 1 and Fig 2 show the ability of Nephronin to inhibit LPS induced TNF-α and IL-1α production.

2. The synthesised Nephronin binds to Heparin and inhibits its migration on cellulose acetate electrophoresis.

The isolated Nephronin was shown to bind specifically to Heparin and Heparan sulphate but not other glycosaminoglycans. To demonstrate that the synthesised Nephronin behave similarly, different preparations of this compound were tested and shown to inhibit the migration of Heparin on cellulose acetate electrophoresis.

3. The synthesised Nephronin inhibits Concanavalin A and PHA stimulation of human lymphocytes.

Concanavalin A (ConA) and PHA were used as positive controls in these studies. Interestingly, Nephronin inhibited the activation of PBMC by both ConA (5μg/ml) and PHA (μg/ml). Fig 3 and Fig 4 show the inhibition of PHA induced cytokine production by Nephronin. It is noteworthy that both Con A and PHA activate PBMC by cross-linking specific sugar moieties on the cell surface.

The specificity of Nephronin for heparin and heparan sulphate had been previously demonstrated. However, the fact that Nephronin can also inhibit stimulation of PBMC by ConA and PHA is of particular importance. Presently I am exploring this aspect of the project.

4. The synthesised Nephronin inhibits stimulation of lymphocytes by streptococcal membrane protein (M-protein), and streptococcal toxin (superantigens) Spe A and Spe B.

Given the fact that Nephronin is also able to inhibit the effects of Con A and PHA, it was imperative to demonstrate other biological activities that Nephronin might have. For this reason, streptococcal membrane protein (M-protein) and streptococcal toxins (superantigens) Spe A and Spe B were used as positive controls.

Endotoxin exerts its biological activity by binding to a serum protein; endotoxin binding protein (LBP). This complex then binds to CD14 on the surface of macrophages and results in the stimulation of cells and release of cytokines.

Gram-positive bacterial toxin (superantigen) activates cells in an entirely different pathway to that of endotoxin. They activate lymphocytes by binding to the outer cleft of the major histocompatibility complex (MHC) and the T cell receptor (TCR). These data suggest that Nephronin specifically inhibit the toxic effects of endotoxin. However, the fact that Nephronin also inhibits activation of lymphocytes by streptococcal bacterial toxins and membrane protein, suggests that Nephronin specifically binds to the cell surface of cells and inhibiting binding of these mitogens to the cells. Alternatively, since these toxins and membrane proteins are closely associated with hyaluronic acid, it is possible that Nephronin binding of Nephronin to these moieties inhibits the mitogenic activity of these toxins and membrane proteins.

The reason that streptococci membrane proteins and toxins were used is due to availability. Attempts in obtaining staphylococcus enterotoxin for these studies have proved very difficult. However, future studies are required when highly purified preparations are available.

5. **Nephronin inhibits adhesion of pathogenic *E.coli* to a gut cell line and prevents bacterial invasion of cells.**

An experimental model to investigate the invasion of pathogenic *E.coli* was utilised to study the effect of Nephronin. In this study, *E.coli* is added to a gut cell line and after a timed incubation period, the bacterial invasion of the cells is determined.

Addition of Nephronin to the cells inhibited bacterial adhesion to the cell surface and invasion of the cells by over 60%. Interestingly, addition of Nephronin to bacteria alone did not inhibit bacterial growth, suggesting that Nephronin does not have a bactericidal cticity.

6. **<u>Inhibition of Herpes Simplex Virus infection.</u>**

Herpes virus adheres to cells first by binding to heparan sulphate on the cell surface, prior to entry into the cells. Addition of Nephronin to keratinocytes 24h prior to inoculation of cells with HSV inhibited HSV infection by 25-30%. However, when Nephronin was added simultaneously as the virus inoculum, it inhibited virus infectivity by 100%. This suggests that pre-incubation of cells with Nephronin might result in the uptake of Nephronin by the cells and subsequent replacement of heparan sulphate. However, since HSV binds to heparan sulphate, it is likely that Nephronin can bind directly to HSV and

prevent its binding to heparan sulphate on the cell surface. This makes Nephronin a prime candidate for combating HSV and other infective agents that involve binding to heparan sulphate in their disease mechanism.

7. HIV virus infectivity.

On the basis that Nephronin binds to heparin and heparan sulphate as well as other sugar moieties on the cell surface, suggested that Nephronin might interfere with binding of infective agents to the cell surface, thus inhibiting the progression/onset of disease.

HIV virus binds to cells first via GP120 molecule. It is postulated that, Nephronin might either bind directly to GP120 or receptors of GP120 on the ell surface, thus inhibiting HIV infectivity. Human macrophages and monocytes were used in these studies.

Finally, since Nephronin potently inhibits production of cytokines such as TNF-α, IL-1α, it can potentially be used as therapeutic agent in diseases that involve these cytokines, such as rheumatoid arthritis.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this twenty-first day of December 1998

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Table 1. Endotoxin Neutralising Activity of the Factor.

		Additions	OD @ 405nm
Positive Control	Proenzyme	Endotoxin	1.2
Negative Control	Proenzyme	Pyrogen Free water	0.3
Sample	Proenzyme	Endotoxin + Nephronin	0.02

The values shown in the table are a set of representative data obtained using the LAL assay. Where pyrogen free water was used for negative control studies, there is persistent low level activation of the pro-enzyme as shown by the relatively high background values. This is thought to be due to minor contamination with endotoxin. Nephronin clearly very effective in neutralisation of the endotoxin.

Table 2. Binding of Nephronin to gram-negative endotoxin and its agglutinating effect.

5µl of the organisms were mixed with either 5µl of Nephronin or 5µl of PBS on a glass slide. After mixing the cells, the time taken for the agglutination of the cells were recorded. Where the factor was added, agglutination occurred within 2 minutes. For control studies, gram-positive bacteria; *S. aureus* and S. *epidermidis* were used. These studies were carried out using either heat killed and/or penicillin killed or viable organisms.

	Viable Organisms		**Heat and/or penicillin killed**	
Organisms	Organism + PBS	Organism + Nephronin	Organism + PBS	Organism + Nephronin
E. coli	-	+	-	+
Pseudomonas aeruginosa	-	+	-	+
N. meningitidis				
A	-	+	-	+
B	-	+	-	+
W135	-	+	-	+
S. aureus	-	-	-	-
S. epidermidis	-	-	-	-

-. No agglutination was observed

+. Agglutination observed

It is important to point out that viable gram-negative bacteria not always agglutinated with Nephronin. Agglutination could be achieved by using a higher concentration of Nephronin.

Table 3. Identification of cells of origin for the production of Nephronin.

Peripheral Blood Mononucleocytes (PBMC) were isolated from SRNS patients and Normal Subjects.
The cells were cultured in medium containing IL2 to maintain the cells.

	Source	Additions	Assay for Nephronin
PBMC	SRNS	Nil	Positive
PBMC	Normal	Nil	Negative
PBMC	Normal	10% SRNS Plasma	Positive
PBMC	Normal	10% Normal Plasma	Negative
PBMC	Normal	Nephronin	Positive

The studies above clearly demonstrate that the PBMC from SRNS patients were capable of producing this material and that sera from these patients stimulated PBMC isolated from healthy subjects to produce the factor. These PBMC were cultured for periods of up to 72 hours and at 24 hour intervals the culture media were removed and tested for the production of the Nephronin.

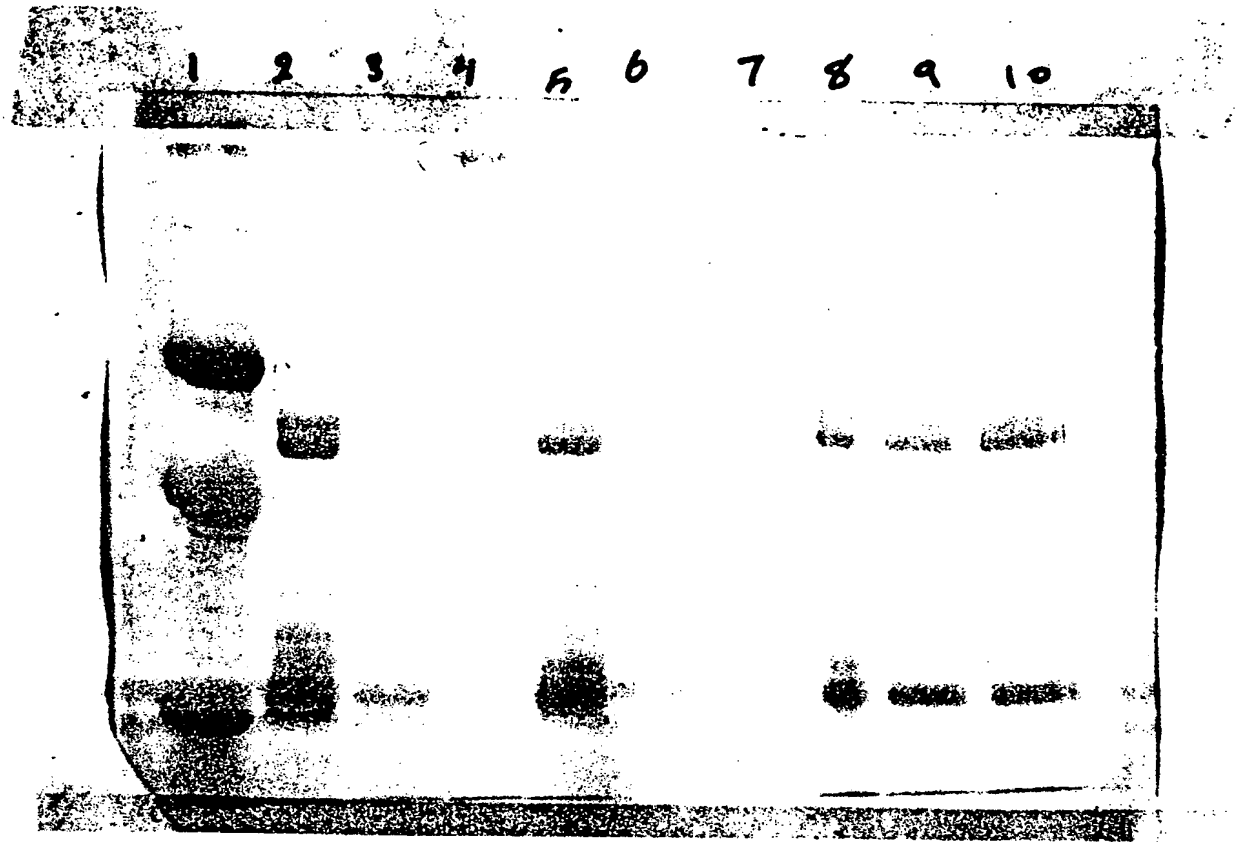
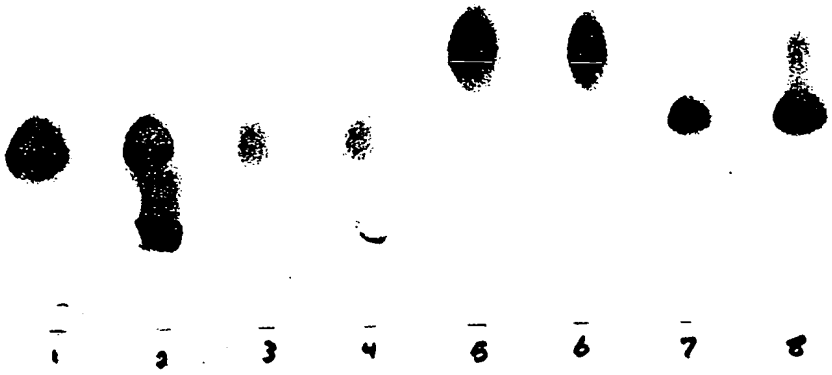


Fig 1. SDS-PAGE pattern of the so called cationic protein purified from the urine of children with Steroid Responsive Nephrotic Syndrome (SRNS), subsequently found to be immunoglobulin.

Lane 1, Molecular weight standards; lanes 2 and 3, two major bands under reducing conditions from two different preparations; lanes 4 and 7, pattern of the protein bands observed under non-reducing conditions; lanes 5, 8, 9 and 10, pattern of the proteins observed under reducing conditions from different preparations and patients urine. Lane 6, urine sample from normal subject, processed as for the SRNS patient's samples.



1- Heparin
2- Heparin + Factor
3- Heparan Sulphate
4- Heparan Sulphate + Factor
5- Chondroitin Sulphate
6- Chondroitin Sulphate + Factor
7- Dermatan Sulphate
8- Dermatan Sulphate + Factor

Fig 2. Cellulose acetate electrophoresis of heparin, heparan sulphate, chondroitin sulphate and dermatan sulphate in the presence and the absence of the factor.
In each case, 0.5 µl of a 2 mg/mL stock solution of each sample was applied to the cellulose acetate. Where the factor was used, 2 µl of the factor was premixed with 0.5 µl of the appropriate glycosaminoglycan and the total sample mixture was then loaded onto the cellulose acetate.

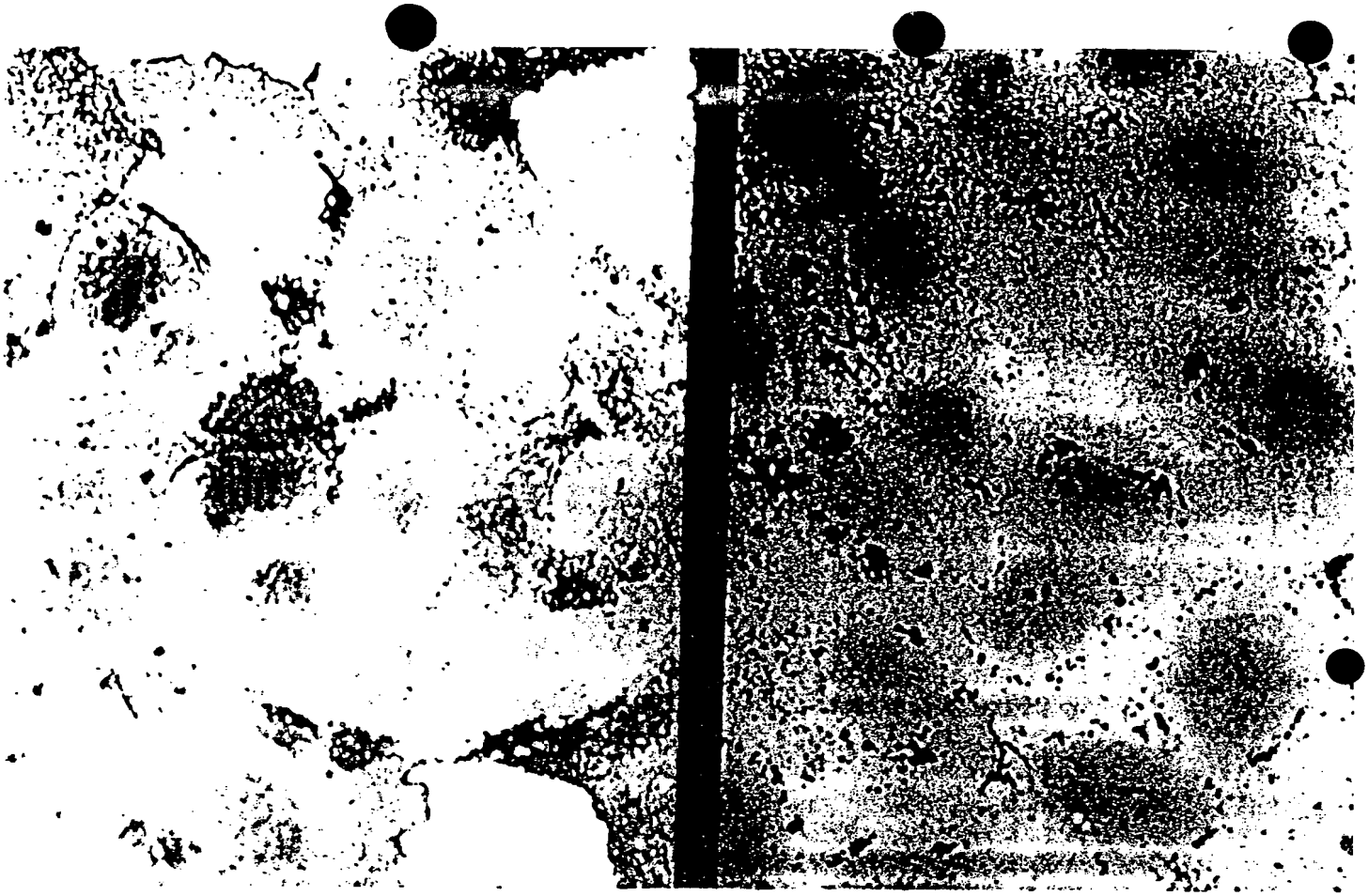


Plate A

Plate B

Fig 3. Gold conjugated poly-L-lysine staining of human umbilical vein endothelial cells.

The endothelial cells were grown to confluence on gelatinised cover-slips. The cells were washed in PBS and then fixed with 4% paraformaldehyde. To specifically label cell surface heparan sulphate, the poly-l-lysine probe was incubated with the cells at pH 1-1.5. The cells were counterstained with Meyer's Haematoxylin for 1 min before mounting in Aquamount.

Plate A shows control staining of the endothelial cells with the cationic probe, poly-l-lysine gold-conjugate.

Plate B shows the HUVECs first incubated with the factor for 60 min. prior to staining with the cationic probe.

Fig 4. Binding of the factor to heparan sulphate on viable endothelial cells.
Endothelial cells were grown to confluence on gelatinised cover-slips. The viable cells were incubated with the factor in the culture medium for 10 min.
The cells were then washed and fixed in cold methanol (-20°C) prior to staining with the cationic probe poly-L-lysine gold-conjugate. The cells were counterstained with Meyer's Haematoxylin for 1 min before mounting in Aquamount.

Plate A shows the control staining for negatively charged molecules on the cell surface of the endothelial cells.

Plate B shows the reduced gold staining of the endothelial cells incubated with the factor in the culture medium.

Fig 4

Plate A

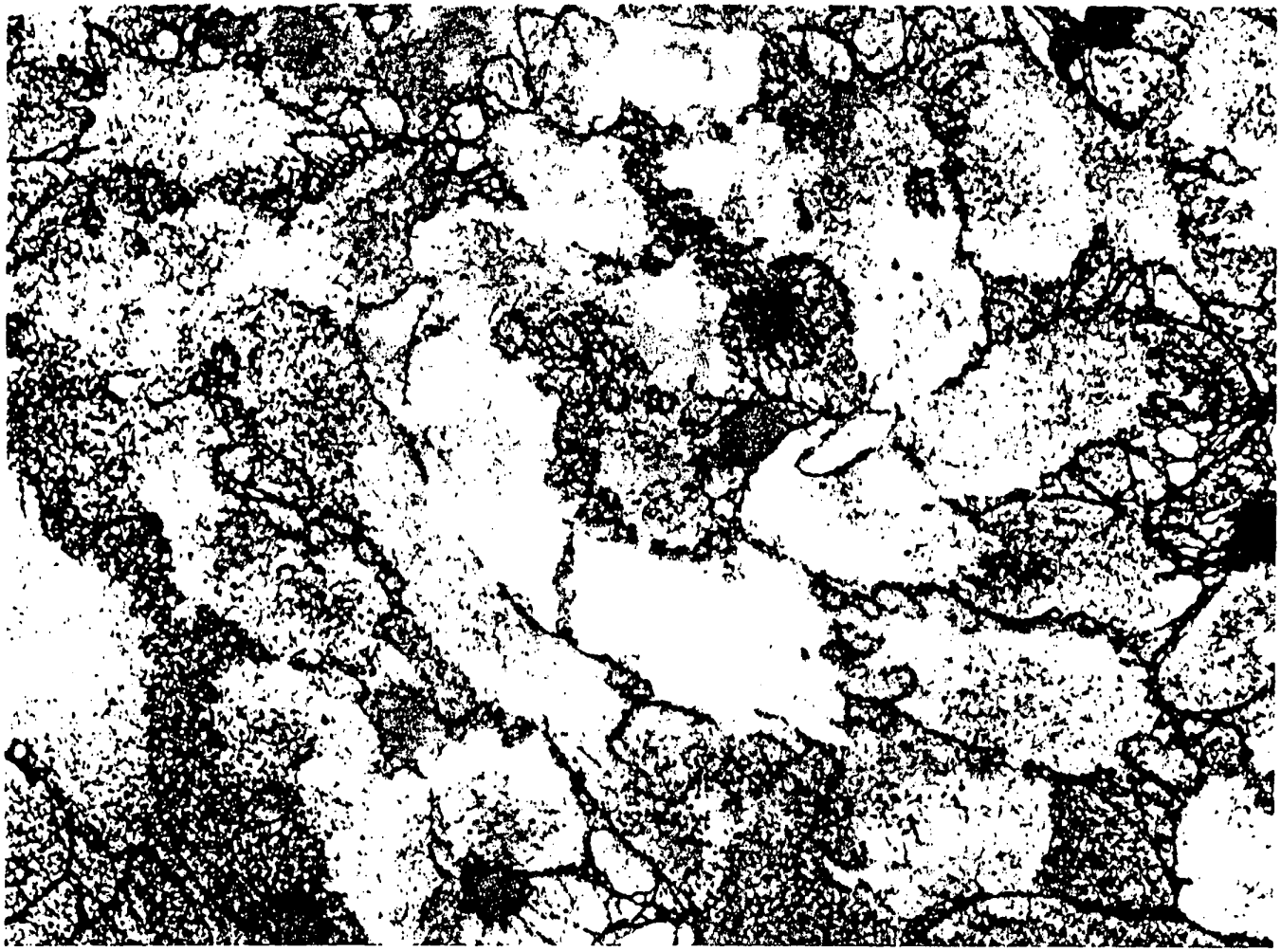


Fig 4

Plate B

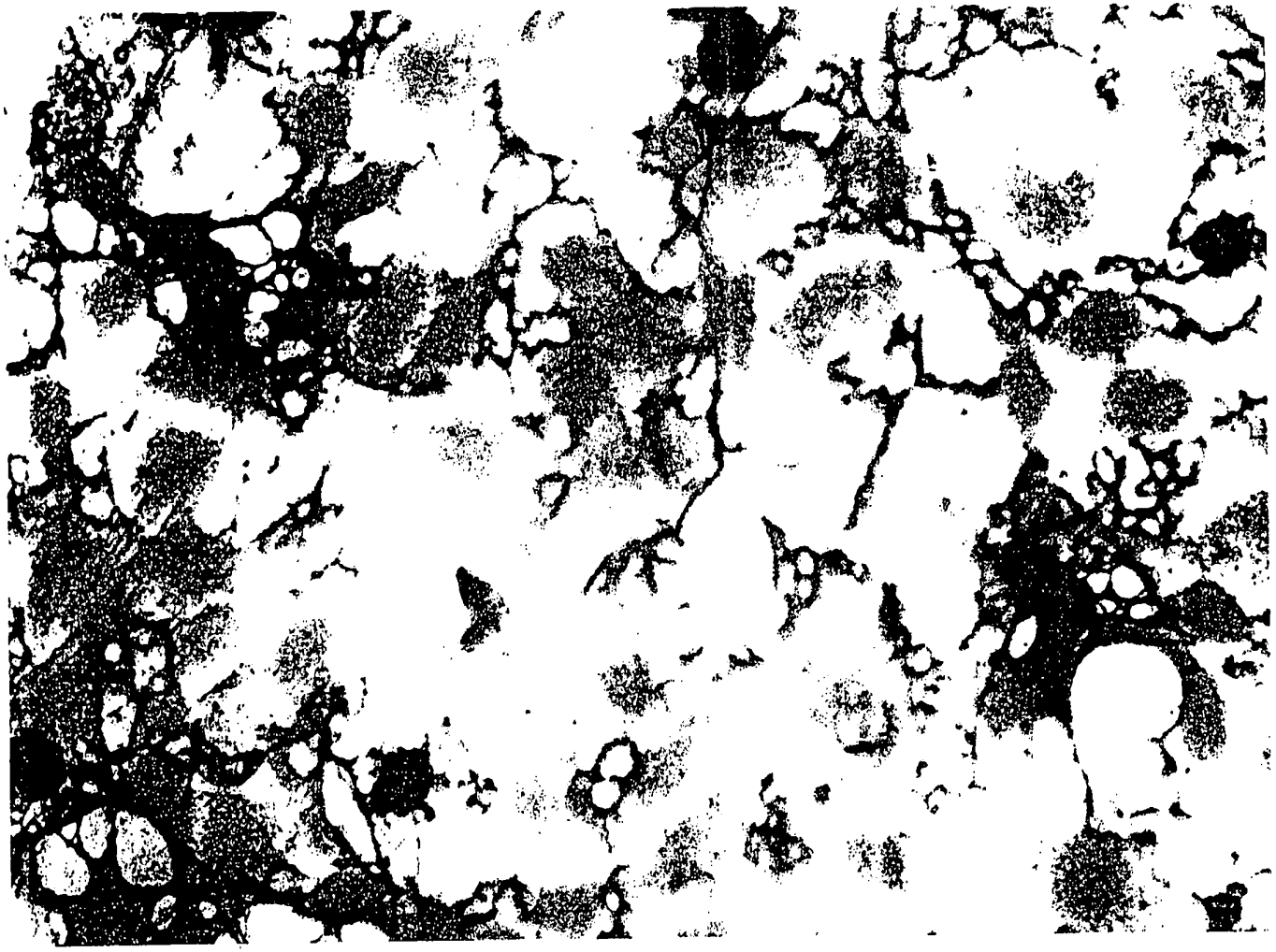


Fig 5. Principle for Limulus Amoebocyte lysate activation.

Coatest Endotoxin assay

Limulus Amoebocyte Lysate.

The proenzyme is activated by Gram-negative bacterial endotoxin.

$$\text{Proenzyme} \xrightarrow{\text{Endotoxin}} \text{Enzyme*} \quad \text{(Activated)}$$

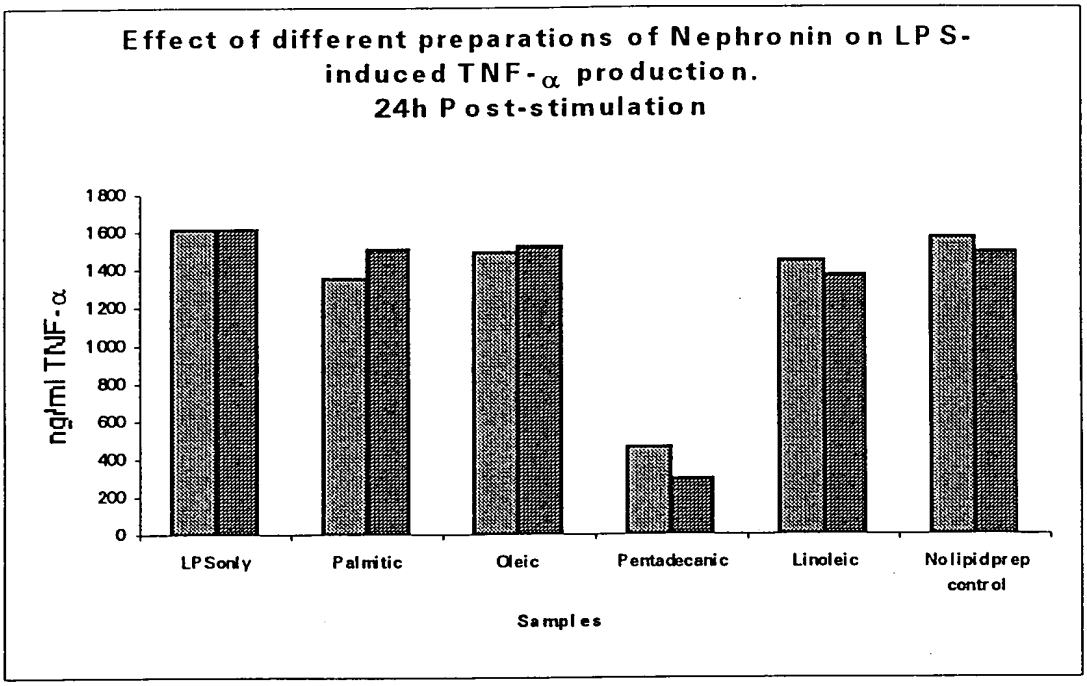
The activated enzyme cleaves off p-nitroaniline (pNA) from the substrate S-2423

$$\text{Substrate} \xrightarrow{\text{Enzyme*}} \text{Peptide + pNA} \quad \text{(yellow)}$$

Measure colour formation at 405 nm.

Experiment 9

Fig 1

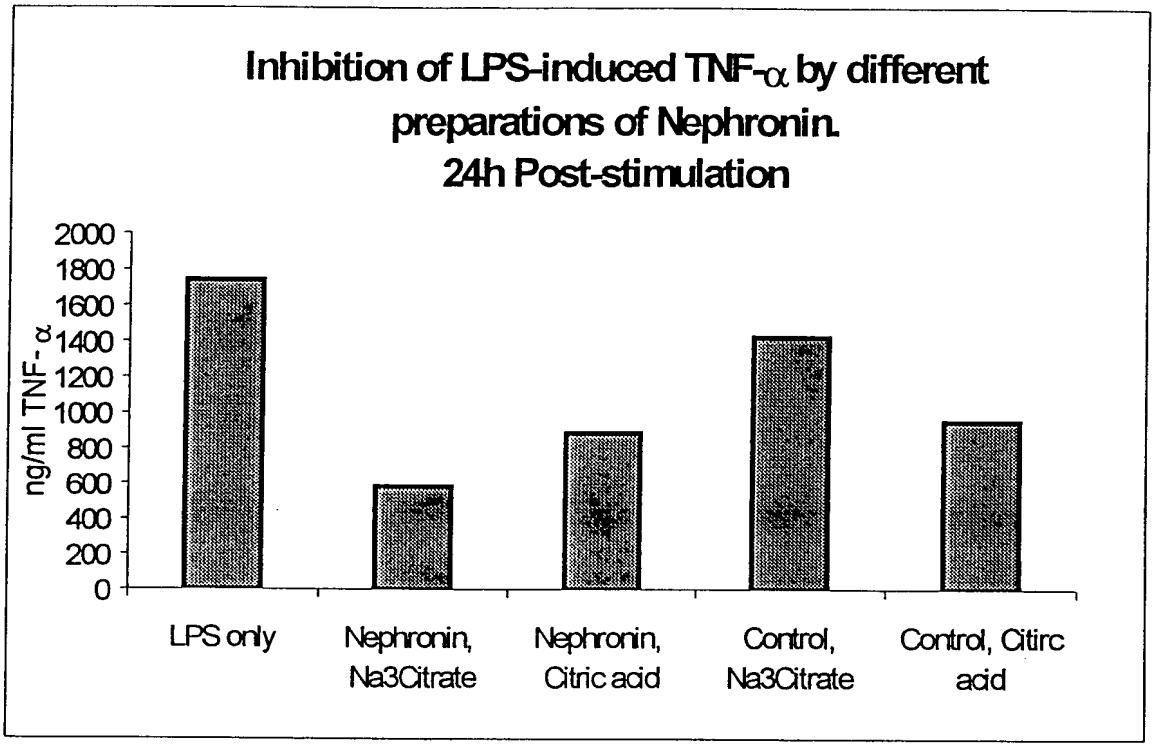


Long chain fatty acids were used in synthesis of Nephronin. The synthesis was performed under nitrogen to reduce oxidation of reactants.

The pH of these preparations was adjusted with 0.2M Na3Citrate. Because of solubility problems, prior to their use in the biological system, these preparations were heated to 50°C so that a homogenous mixture was produced.
Each sample was tested in duplicate except that in one instance the preparations were added to the culture plates containing the LPS and incubated for 1h prior to the addition of the cells. Alternatively, they were added to PBMC pre-mixed with LPS.
The preparations are identified by the name of the acid used.
The control preparation refers to preparation where the synthesis reaction was performed in the absence of fatty acid.

Experiment 12

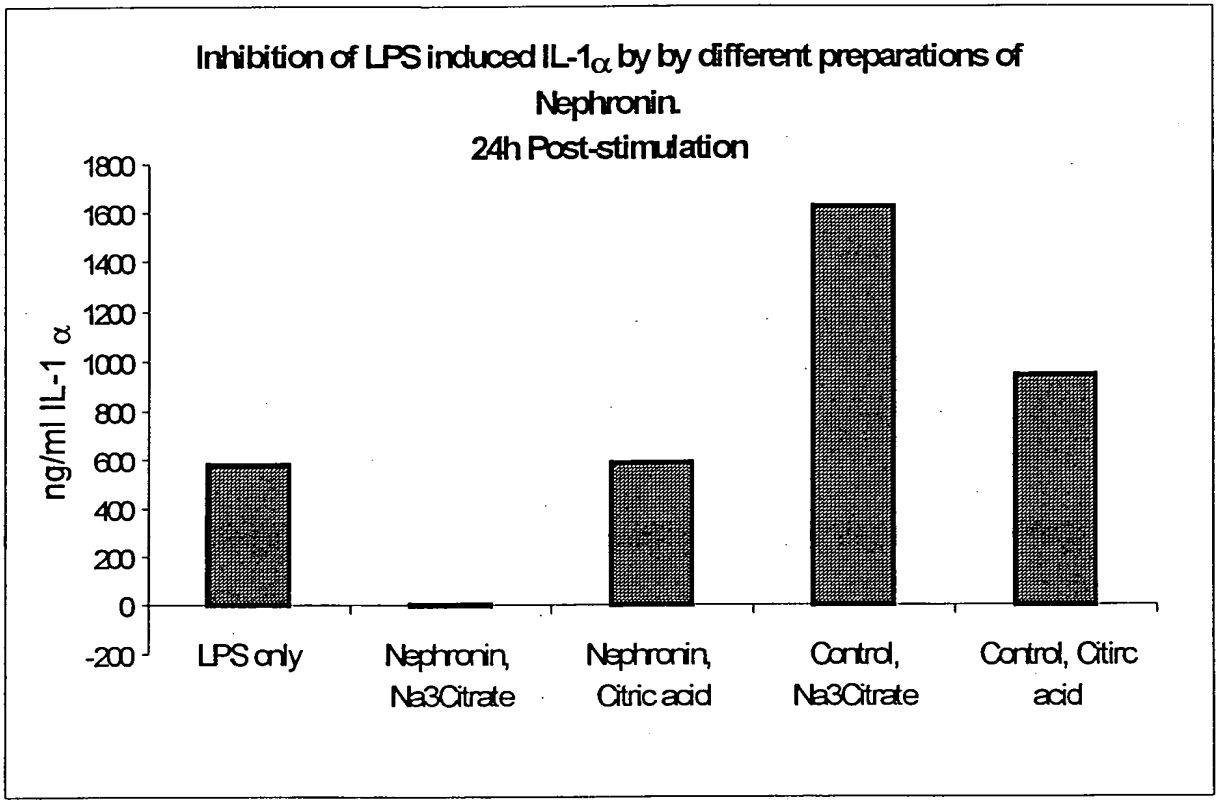
Fig 1



Preparations of Nephronin, dissolved in water and the pH adjusted to neutral with 10% ammonium hydroxide solution.
Each sample was tested for its biological activity with isolated human PBMC.
50µl of each preparation was added to 1ml of PBMC at $1.5x10^6$cells/ml containing 1ng/ml LPS.
The concentration of TNF-α was determined in the culture supernatant 24 h post-stimulation.

Experiment 12

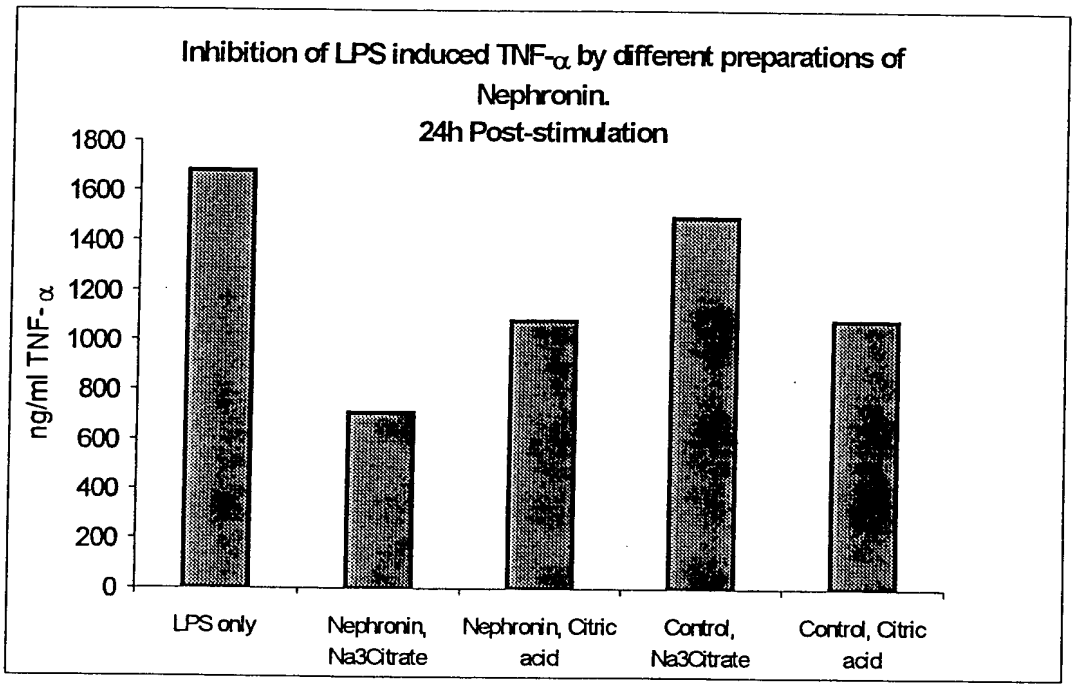
Fig 2



Preparations of Nephronin, dissolved in water and the pH adjusted to neutral with 10% ammonium hydroxide. 50µl of each preparation was added to 1ml of PBMC containing 1ng/ml LPS.
Each sample was tested for its biological activity with isolated human PBMC.
The concentration of IL-1α was determined in the culture supernatants 24h post-stimulation.

Experiment 12

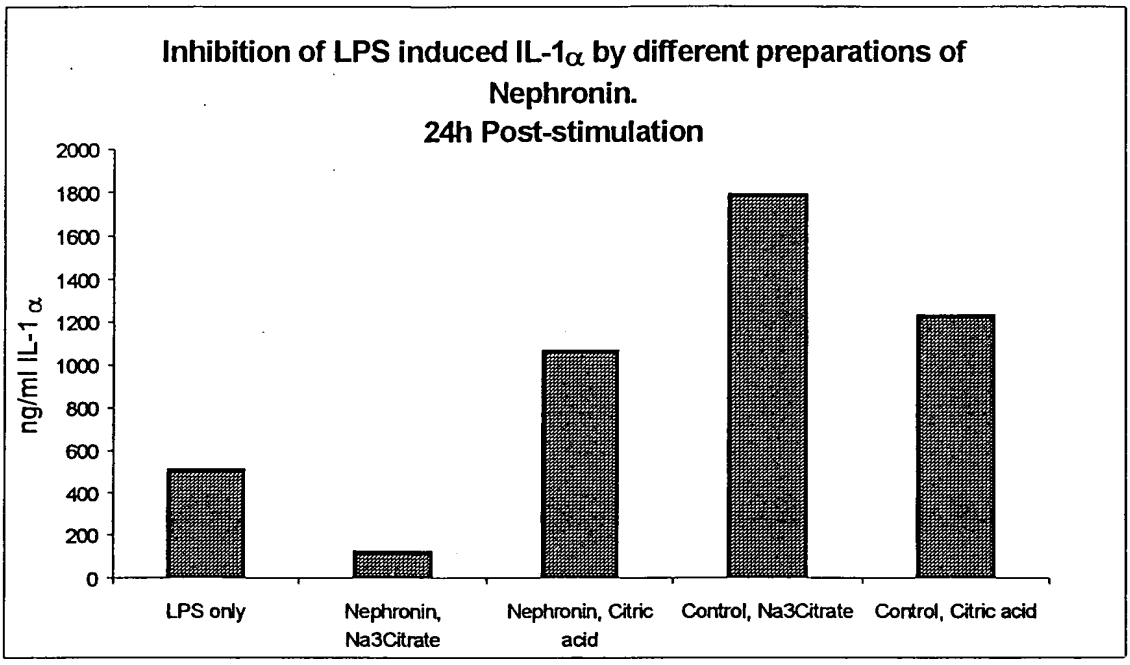
Fig 3.



Preparations of Nephronin, dissolved in water and the pH adjusted to neutral with 10% ammonium hydroxide. 50µl of each preparation was added to 1ml of PBMC containing 1ng/ml LPS.
Each sample was tested for its biological activity with isolated human PBMC.
The concentration of TNF-α was determined in the culture supernatants 48 h post-stimulation.

Experiment 12

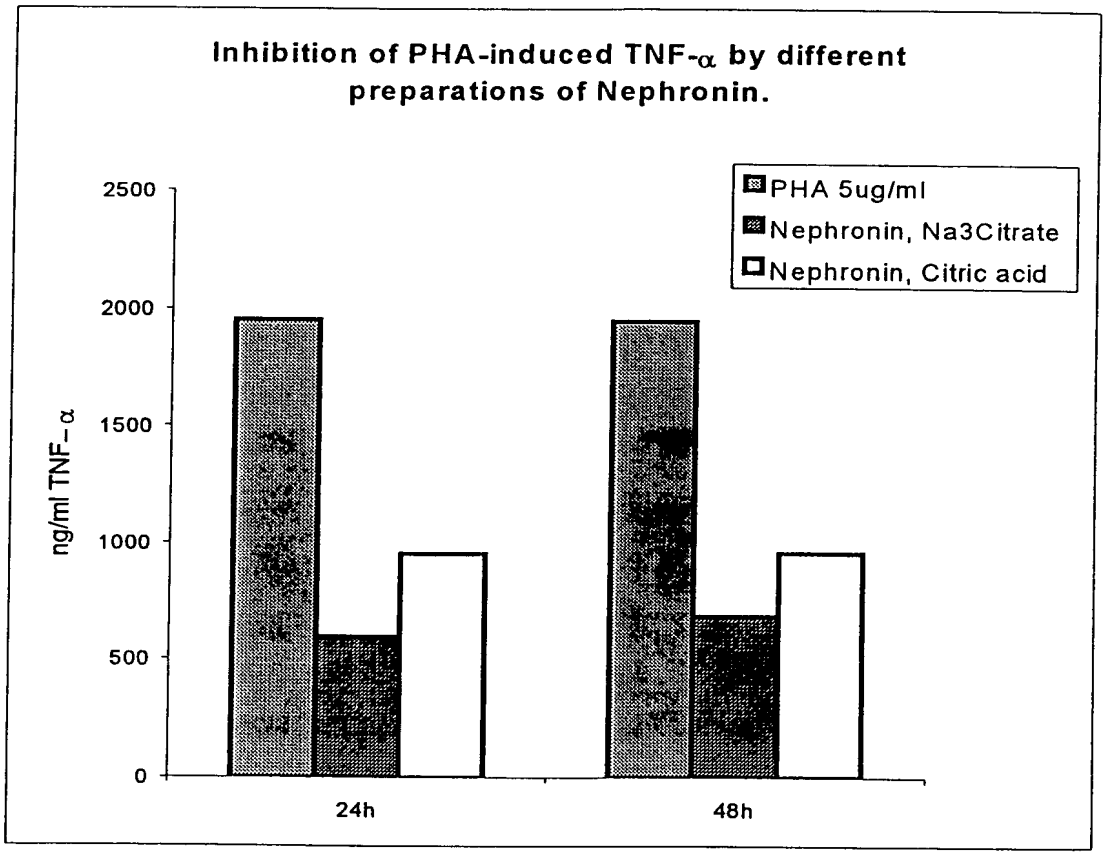
Fig 4



Preparations of Nephronin, dissolved in water and the pH adjusted to neutral with 10% ammonium hydroxide. 50µl of each preparation was added to 1ml of PBMC containing 1ng/ml LPS.
Each sample was tested for its biological activity with isolated human PBMC.
The concentration of IL-1α was determined in the culture supernatant 48 h post-stimulation.

Experiment 12

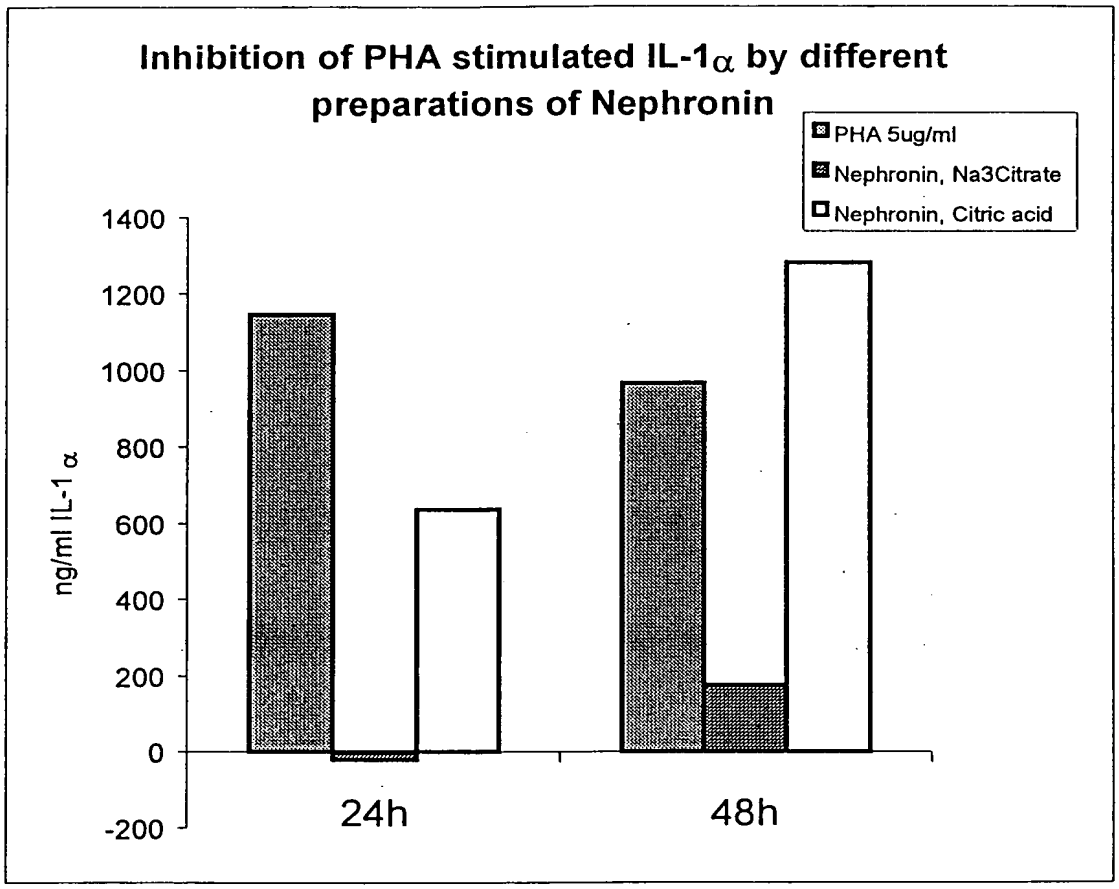
Fig 5



Preparations of Nephronin that were pH adjusted to neutral with 10% ammonium hydroxide solution. In each case 50µl of each preparation was added to human PBMC at 1.5 x 10^6 cells/ml. The cells were stimulated with 5µg/ml of PHA.
The cells were harvested at 24 and 48h post-stimulation and the levels of TNF-α determined.

Experiment 12

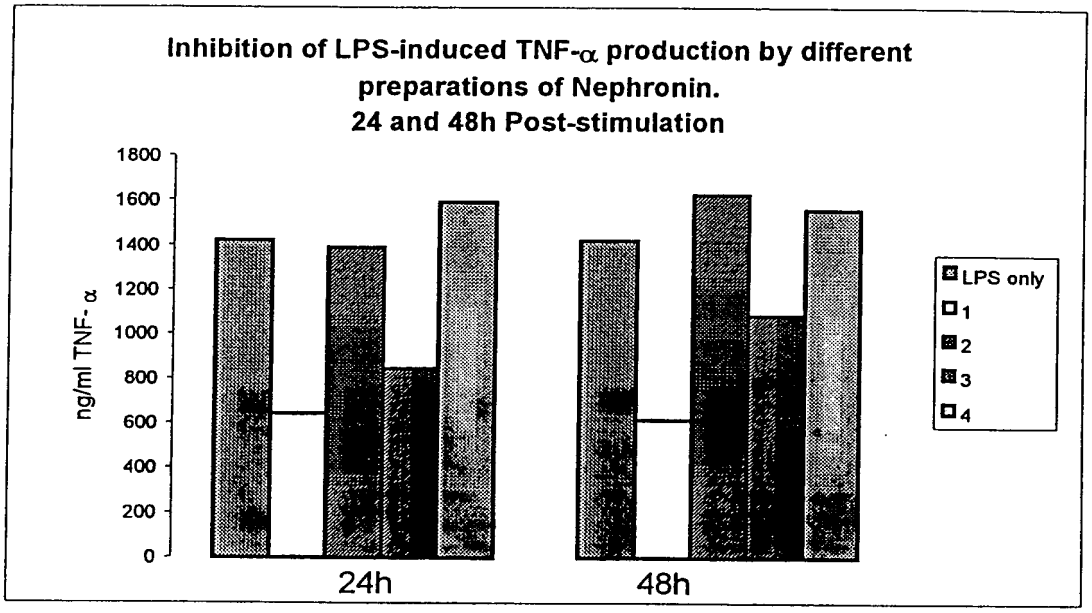
Fig 6



Preparations of Nephronin that were pH adjusted to neutral with 10% ammonium hydroxide solution. In each case 50µl of each preparation was added to human PBMC at 1.5 x 10^6 cells/ml. The cells were stimulated with 5µg/ml of PHA.
The cells were harvested at 24 and 48h post-stimulation and the levels of IL-1α determined.

Experiment 14

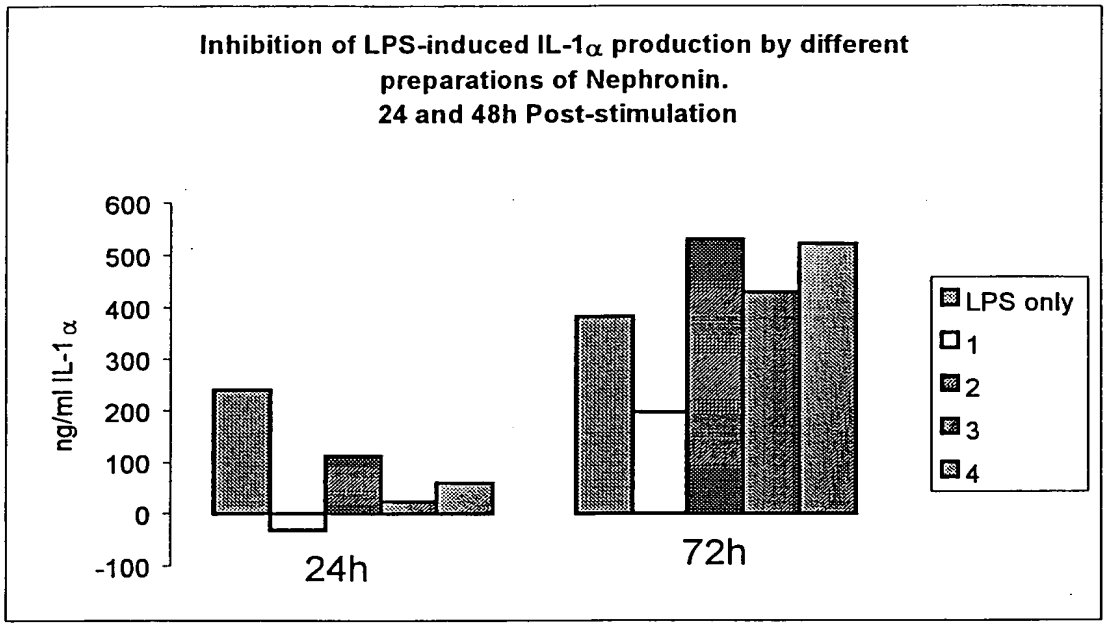
Fig 1



50µl of the preparations of Nephronin that were pH adjusted to Neutral with 10% ammonium hydroxide were tested for their biological activity.
In each case 1ml of PBMC at 1.5x 10^6 cells/ml were used. The cells were harvested at 24 and 48h post-stimulation and the concentration of TNF-α measured by sandwich ELISA.

Experiment 14

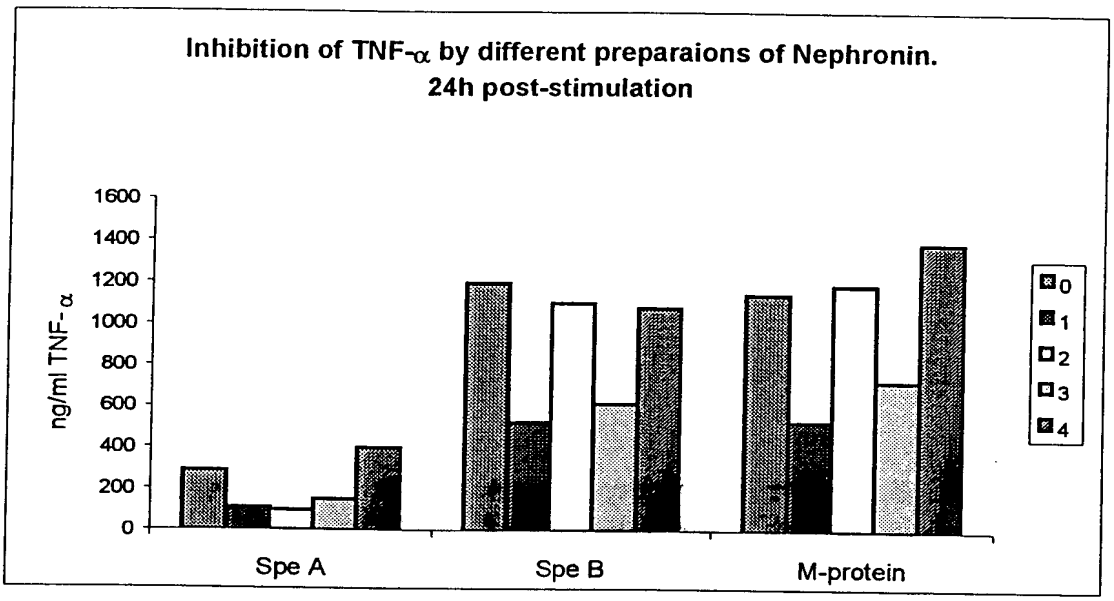
Fig 2



50µl of the preparations of Nephronin that were pH adjusted to Neutral with 10% ammonium hydroxide were tested for their biological activity.
In each case 1ml of PBMC at 1.5x 10^6 cells/ml were used. The cells were harvested at 24 and 48h post-stimulation and the concentration of IL-1α measured by sandwich ELISA.

Experiment 14

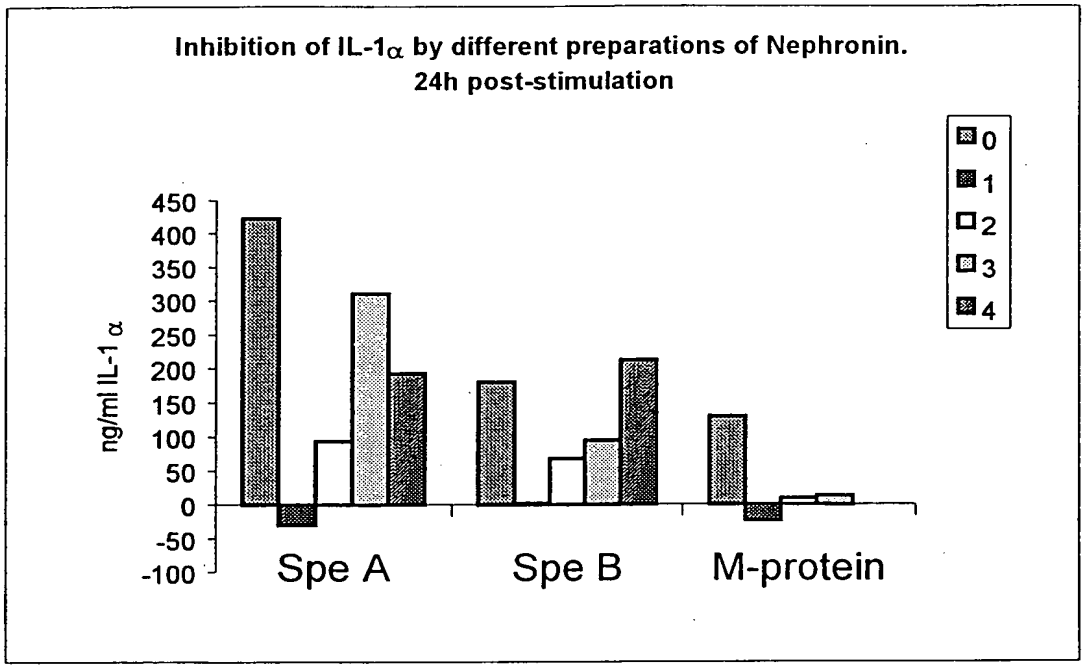
Fig 3



50µl of each preparation of Nephronin, that were pH adjusted to Neutral with 10% ammonium hydroxide, were tested for their biological activity.
In each case 1ml of PBMC at 1.5x 10^6 cells/ml were stimulated with 50 ng/ml of the streptococcal stimulants. The cells were harvested at 24h post-stimulation and the concentration of TNF-α measured by sandwich ELISA.

Experiment 14

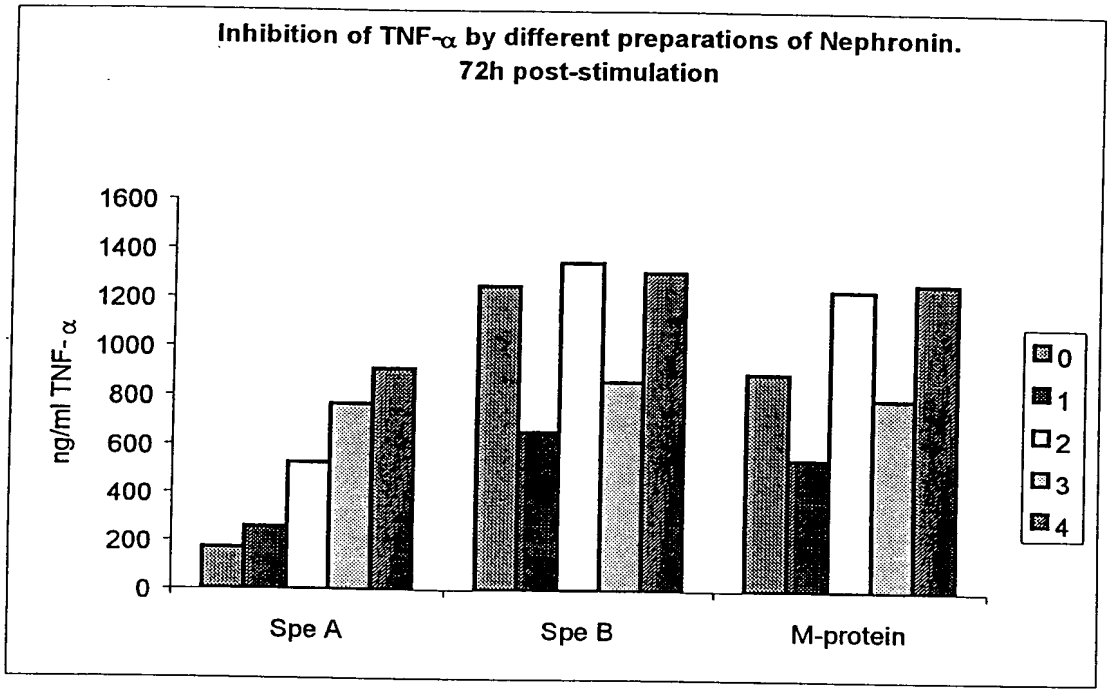
Fig 4



50µl of each preparation of Nephronin, that were pH adjusted to Neutral with 10% ammonium hydroxide, were tested for their biological activity.
In each case 1ml of PBMC at 1.5x 10^6 cells/ml were stimulated with 50 ng/ml of the streptococcal stimulants. The cells were harvested at 24h post-stimulation and the concentration of IL-1α measured by sandwich ELISA.

Experiment 14

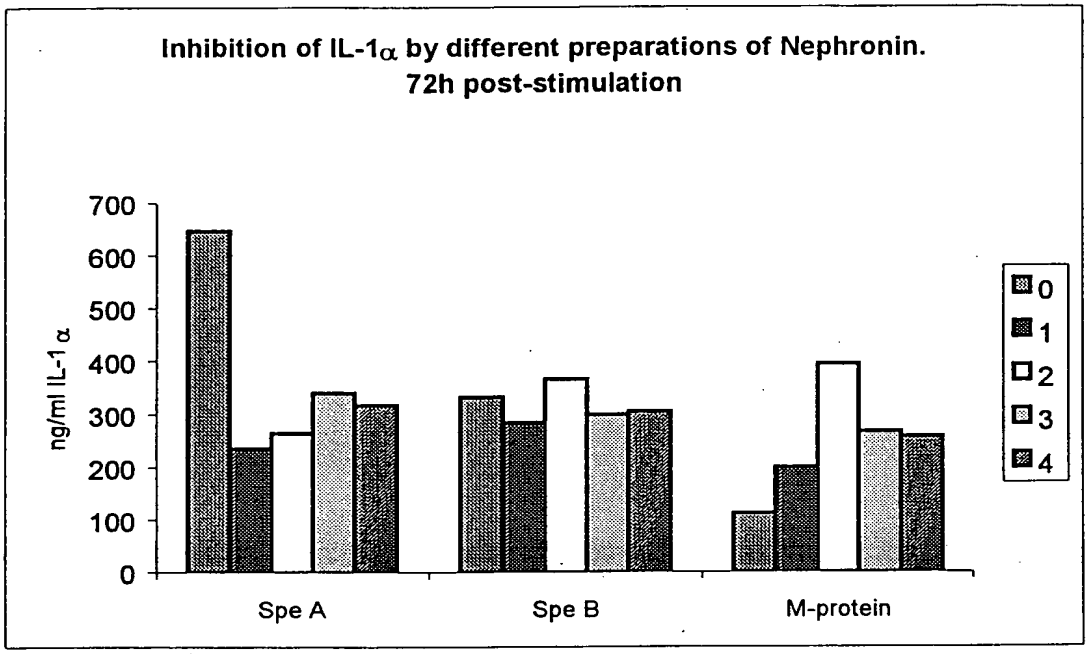
Fig 5



50µl of each preparation of Nephronin, that were pH adjusted to Neutral with 10% ammonium hydroxide, were tested for their biological activity.
In each case 1ml of PBMC at 1.5x 10^6 cells/ml were stimulated with 50ng/ml of the streptococcal stimulants. The cells were harvested at 72h post-stimulation and the concentration of TNF-α measured by sandwich ELISA.

Experiment 14

Fig 6



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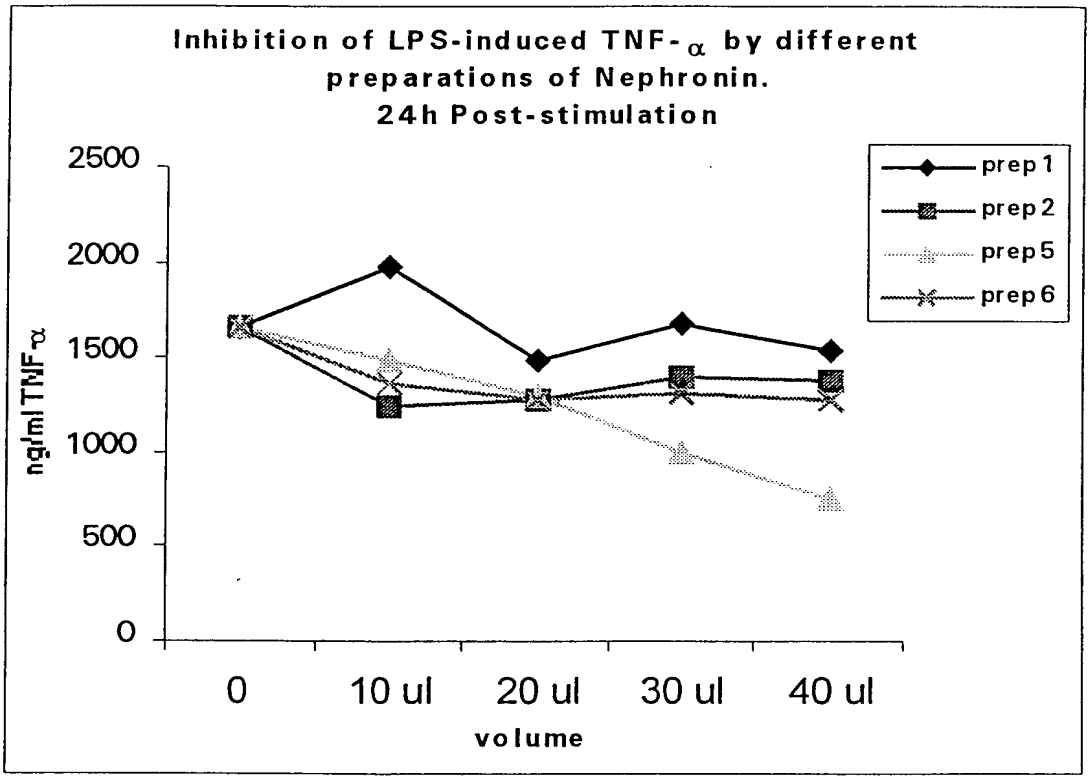
In each case 1ml of PBMC at 1.5x 10^6 cells/ml were stimulated with 50 ng/ml of the streptococcal stimulants. The cells were harvested at 72h post-stimulation and the concentration of IL-1α measured by sandwich ELISA.

Experiment 15

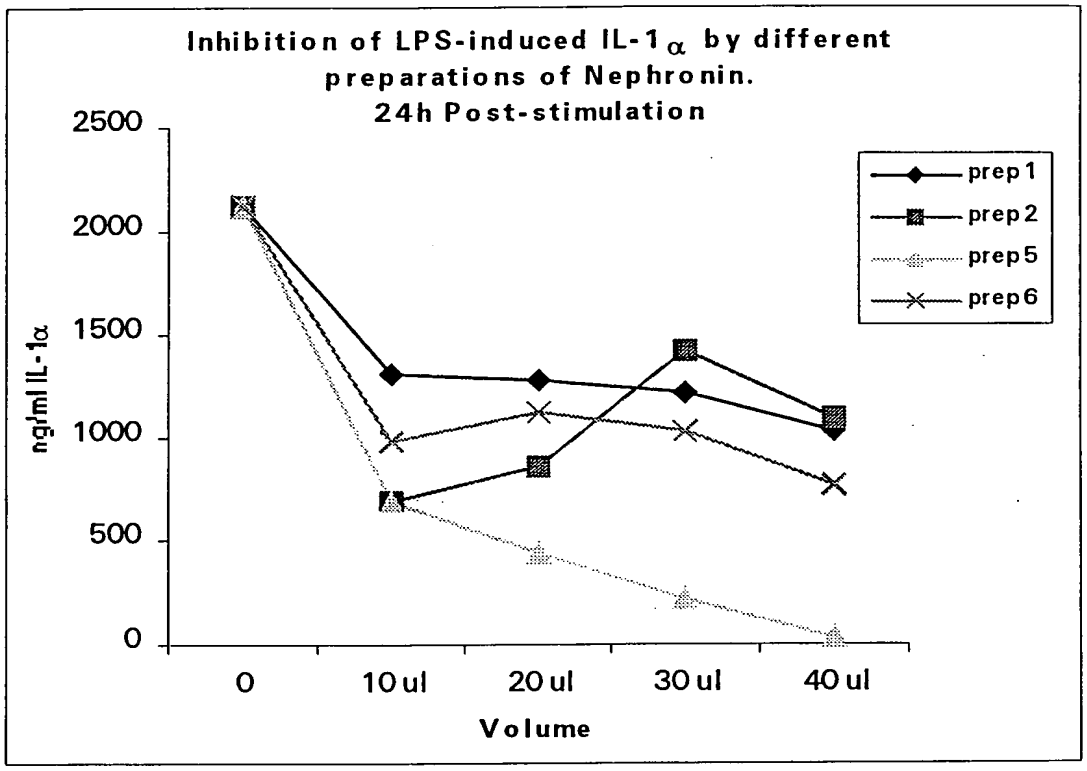
Fig1



Increasing volumes of four preparations of Nephronin were used to investigate their ability to inhibit LPS-induced TNF-α production 24h post-stimulation.
The stated volume of each preparation was added appropriately to chambers of 24 well tissue culture plates. To each well 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS. LPS was added to the cells immediately prior to being added to the culture plates.

Experiment 15

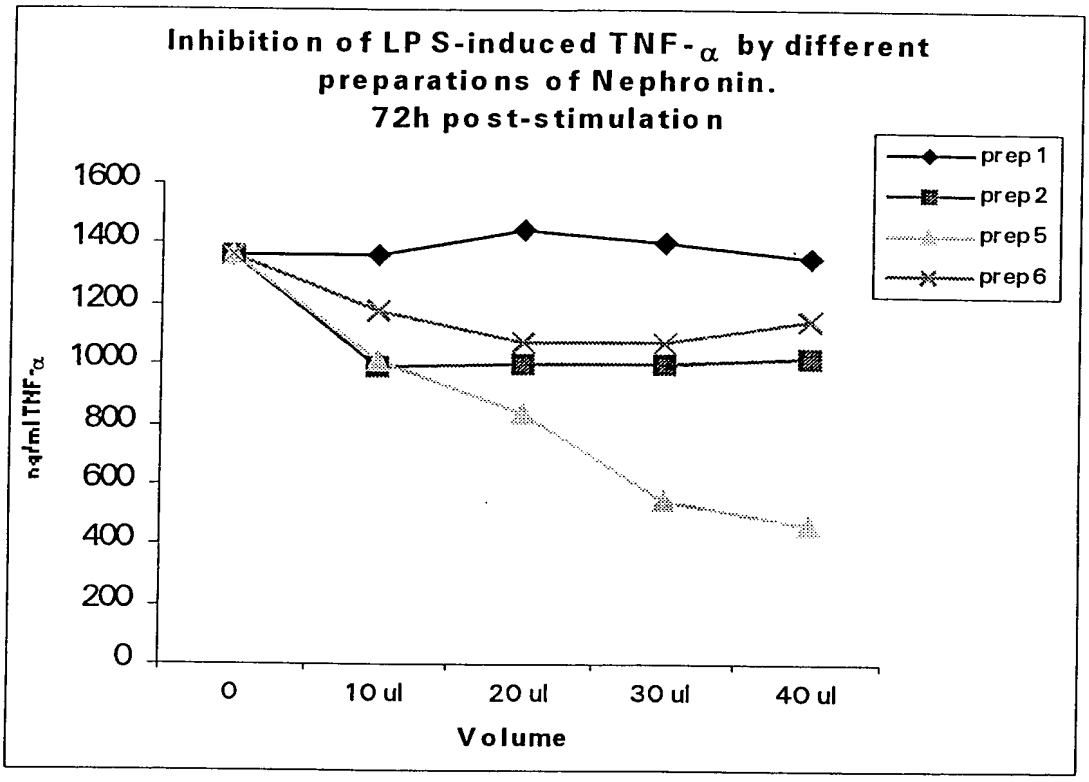
Fig 2



Increasing volumes of four preparations of Nephronin were used to investigate their ability to inhibit LPS-induced IL-1α production 24h post-stimulation.
The stated volume of each preparation was added appropriately to chambers of 24 well tissue culture plates. To each well 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS. LPS was added to the cells immediately prior to being added to the culture plates.

Experiment 15

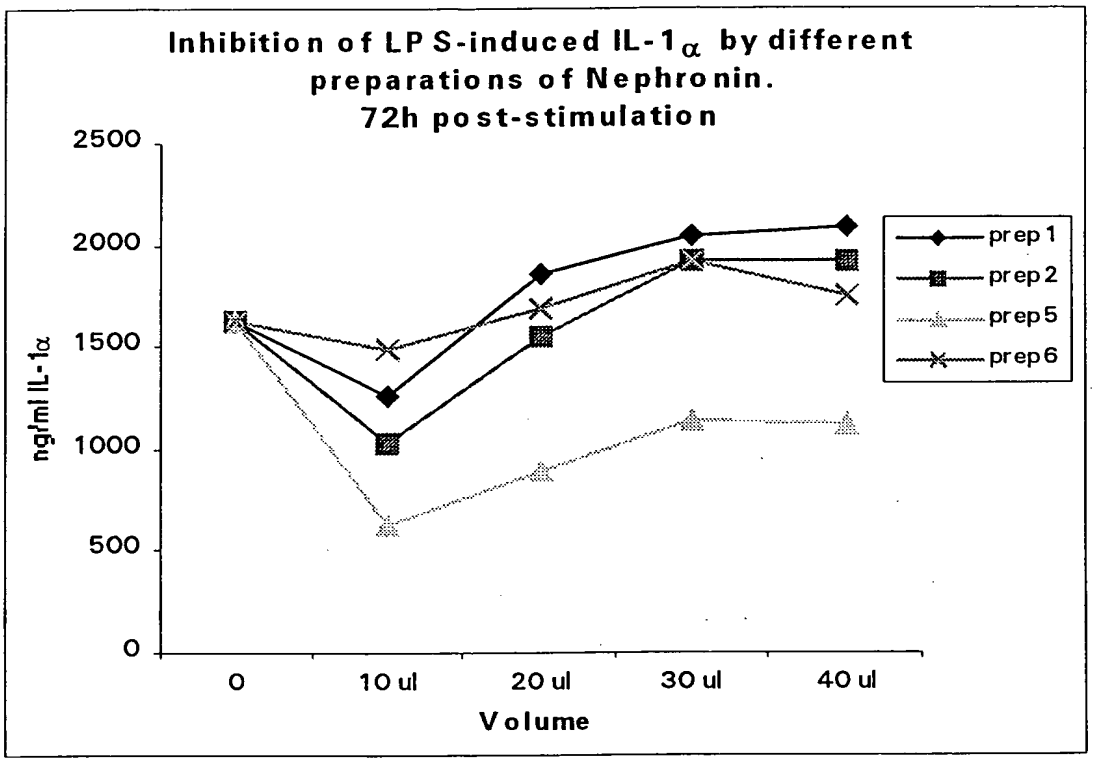
Fig 3



Increasing volumes of four preparations of Nephronin were used to investigate their ability to inhibit LPS-induced TNF-α production 72h post-stimulation.
The stated volume of each preparation was added appropriately to chambers of 24 well tissue culture plates. To each well 1ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS. LPS was added to the cells immediately prior to being added to the culture plates.

Experiment 15

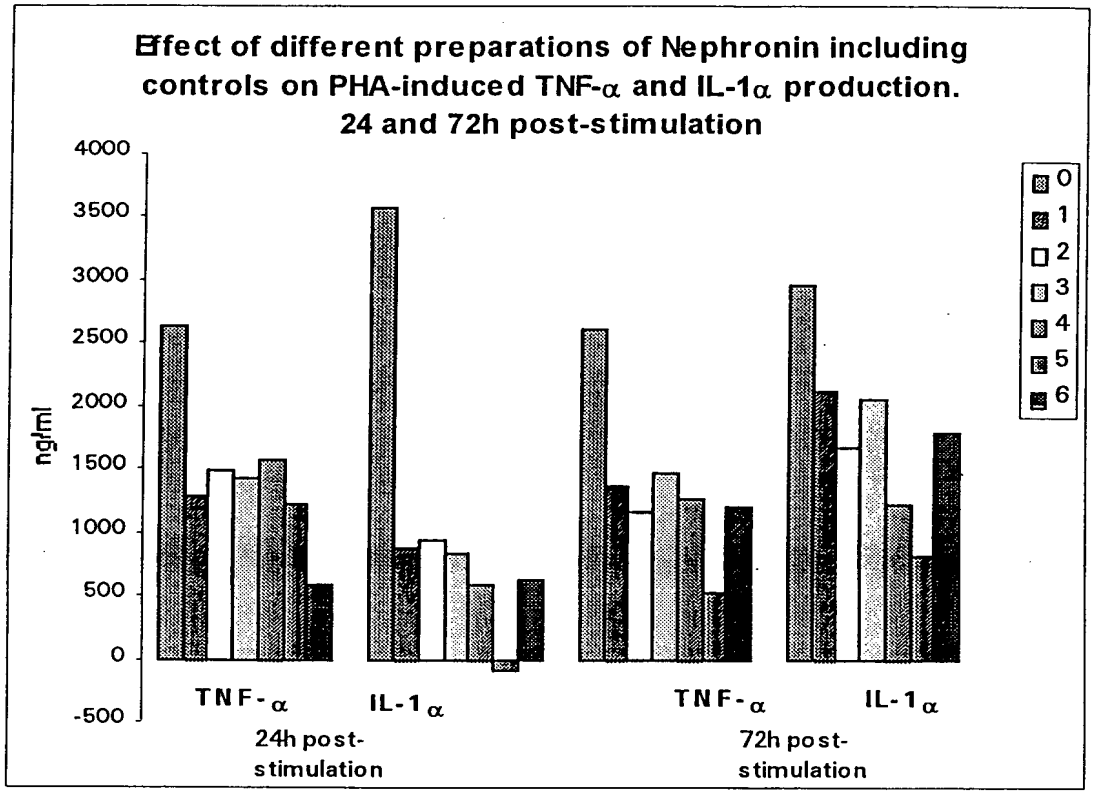
Fig 4



Increasing volumes of four preparations of Nephronin were used to investigate their ability to inhibit LPS-induced IL-1α production 72h post-stimulation.
The stated volume of each preparation was added appropriately to chambers of 24 well tissue culture plates. To each well 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS. LPS was added to the cells immediately prior to being added to the culture plates.

Experiment 15

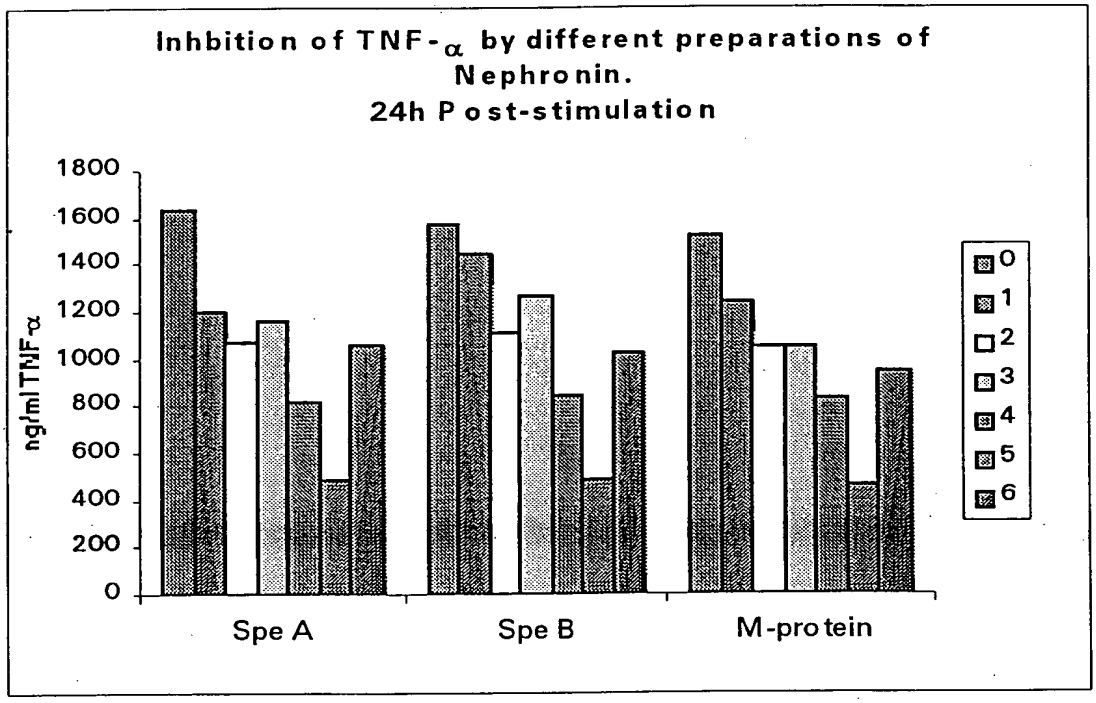
Fig 5



40µl of each preparation was added to chambers of 24 well tissue culture plates. To each well 5µg of PHA was added, followed by 1ml of PBMC at $1.5x10^6$ cells/ml. Cells were harvest at 24 and 72h post-stimulation and the concentration of TNF-α and IL-1α were measured in the culture supernatants.

Experiment 15

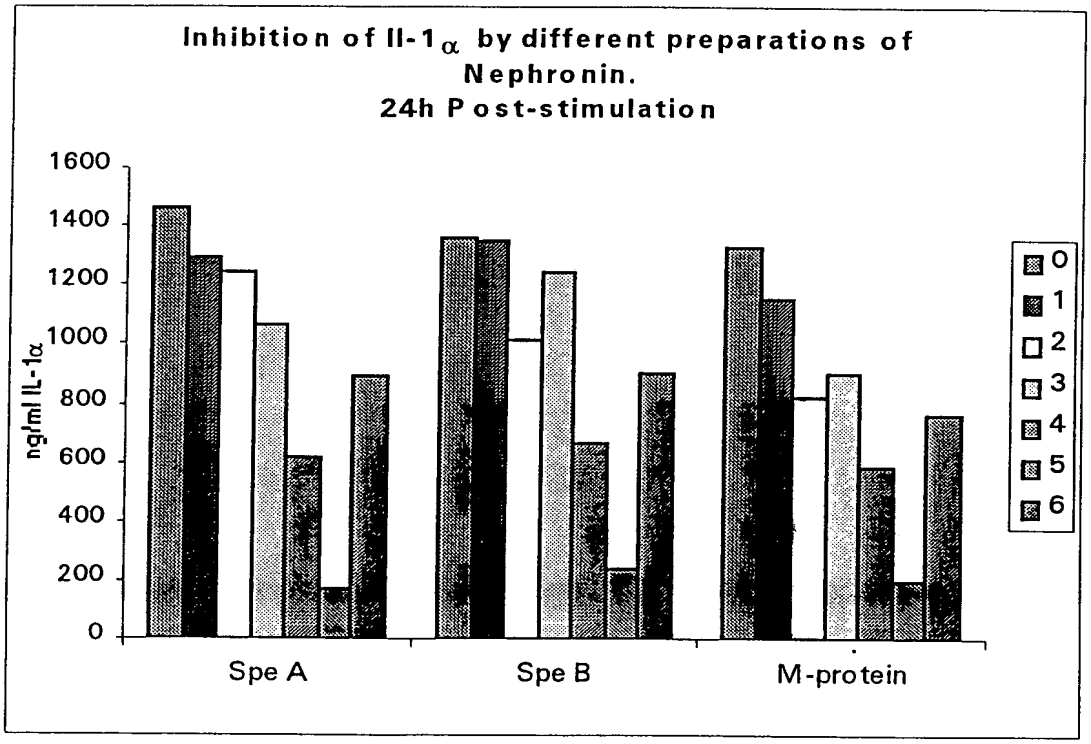
Fig 6



50ng of the stated mitogens were added to chambers of 24 well tissue culture plates. To appropriate wells, 40µl of each preparation was added, followed by 1ml of PBMC at $1.5x10^6$ cells/ml added to each well.
The cells were harvest at 24h post-stimulation and concentration of TNF-α was determined by sandwich ELISA.
0 refers to cells stimulated with the appropriate mitogen only.

Experiment 15

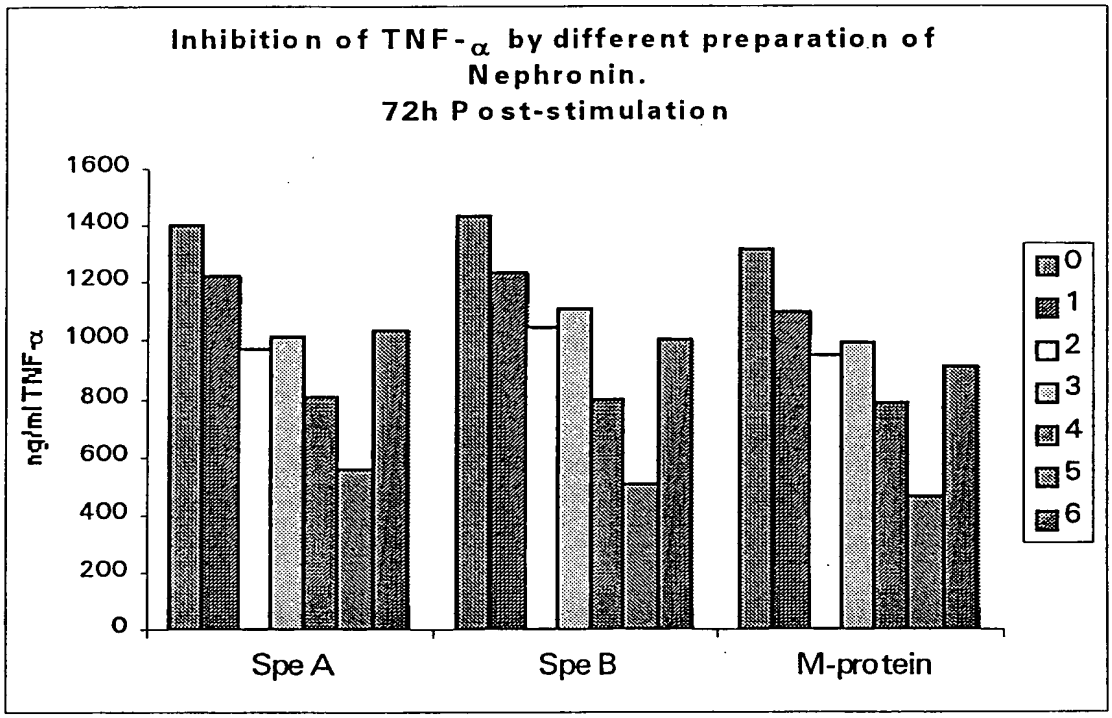
Fig 7



50ng of the stated mitogens were added to chambers of 24 well tissue culture plates. To appropriate wells, 40µl of each preparation was added, followed by 1ml of PBMC at $1.5x10^6$ cells/ml added to each well.
The cells were harvest at 24h post-stimulation and concentration of IL-1α was determined by sandwich ELISA.
0 refers to cells stimulated with the appropriate mitogen only.

Experiment 15

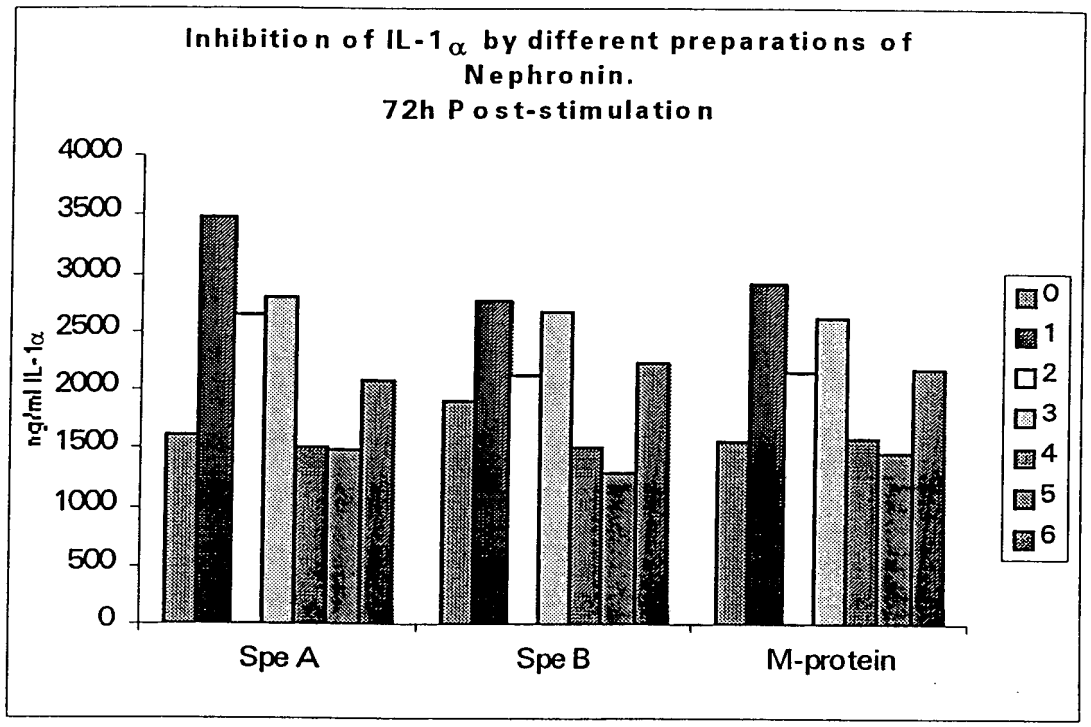
Fig 8



50ng of the stated mitogens were added to chambers of 24 well tissue culture plates. To appropriate wells, 40µl of each preparation was added, followed by 1ml of PBMC at $1.5x10^6$ cells/ml added to each well.
The cells were harvest at 72h post-stimulation and concentration of TNF-α was determined by sandwich ELISA.
0 refers to cells stimulated with the appropriate mitogen only.

Experiment 15

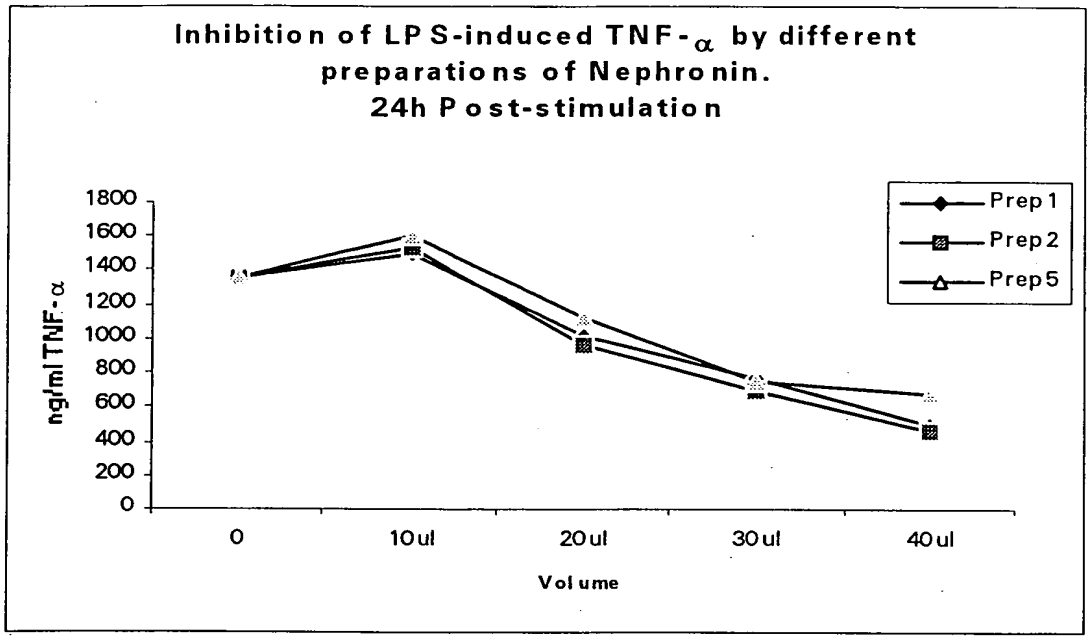
Fig 9



50ng of the stated mitogens were added to chambers of 24 well tissue culture plates. To appropriate wells, 40µl of each preparation was added, followed by 1ml of PBMC at $1.5x10^6$ cells/ml added to each well.
The cells were harvest at 72h post-stimulation and concentration of IL-1α was determined by sandwich ELISA.
0 refers to cells stimulated with the appropriate mitogen only.

Experiment 16

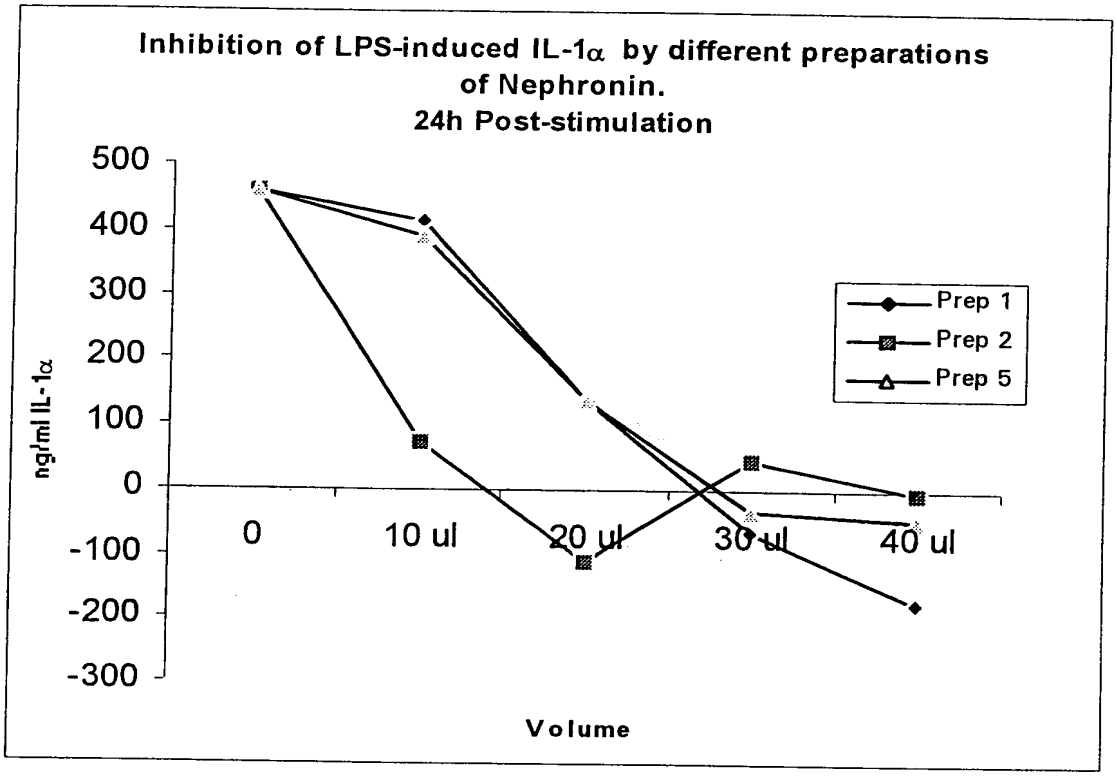
Fig1



Increasing volumes of three preparations of Nephronin were tested for their biological activity.
Preparations of Nephronin were added to chambers of 24 well tissue culture plates.
1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was added to each well.
LPS was added to PBMC immediately prior to the addition of cells.
Cells were harvested 24h post-stimulation and the concentration of TNF-α was measured by sandwich ELISA.

Experiment 16

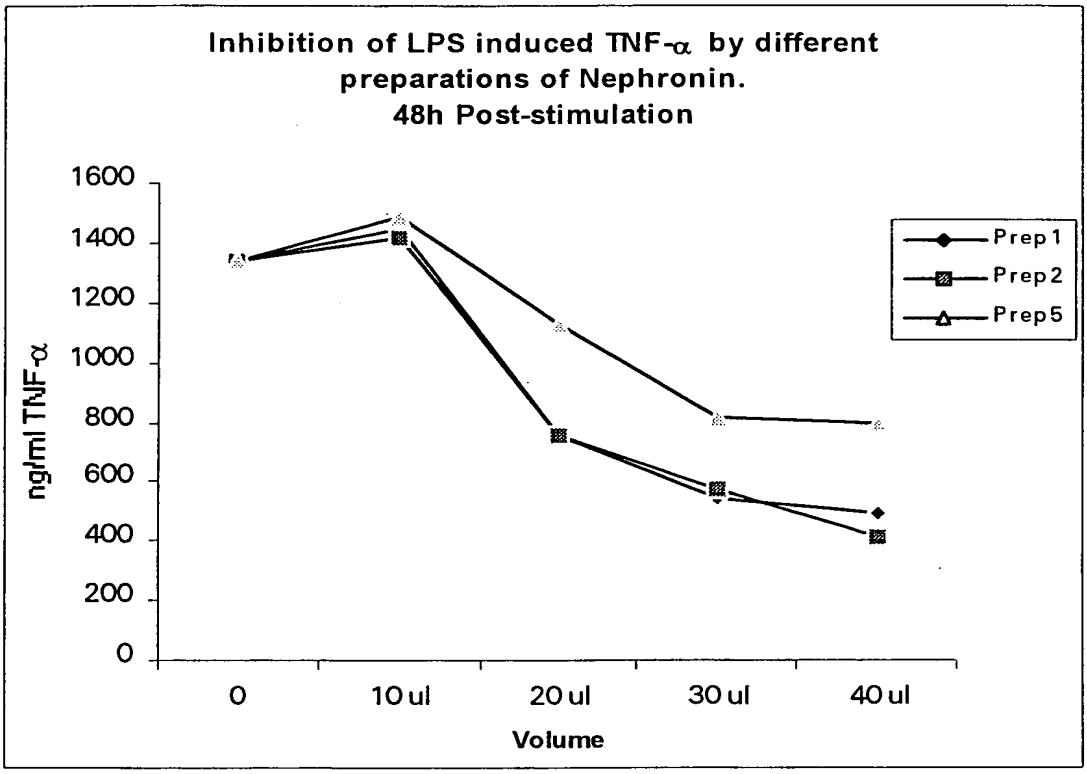
Fig 2



Increasing volumes of three preparations of Nephronin were tested for their biological activity.
Preparations of Nephronin were added to chambers of 24 well tissue culture plates.
1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was added to each well.
LPS was added to PBMC immediately prior to the addition of cells.
Cells were harvested 24h post-stimulation and the concentration of IL-1α was measured by sandwich ELISA.

Experiment 16

Fig 3



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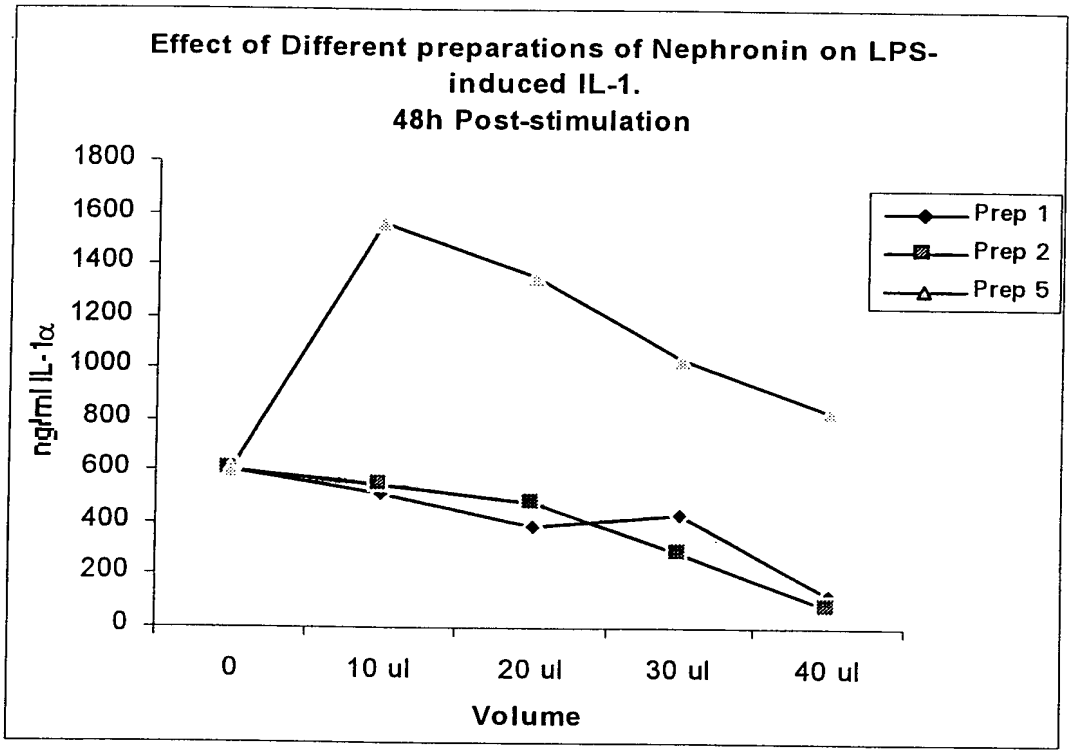
LPS was added to PBMC immediately prior to the addition of cells.
Cells were harvested 48h post-stimulation and the concentration of TNF-α was measured by sandwich ELISA.

Experiment 16

Fig 4



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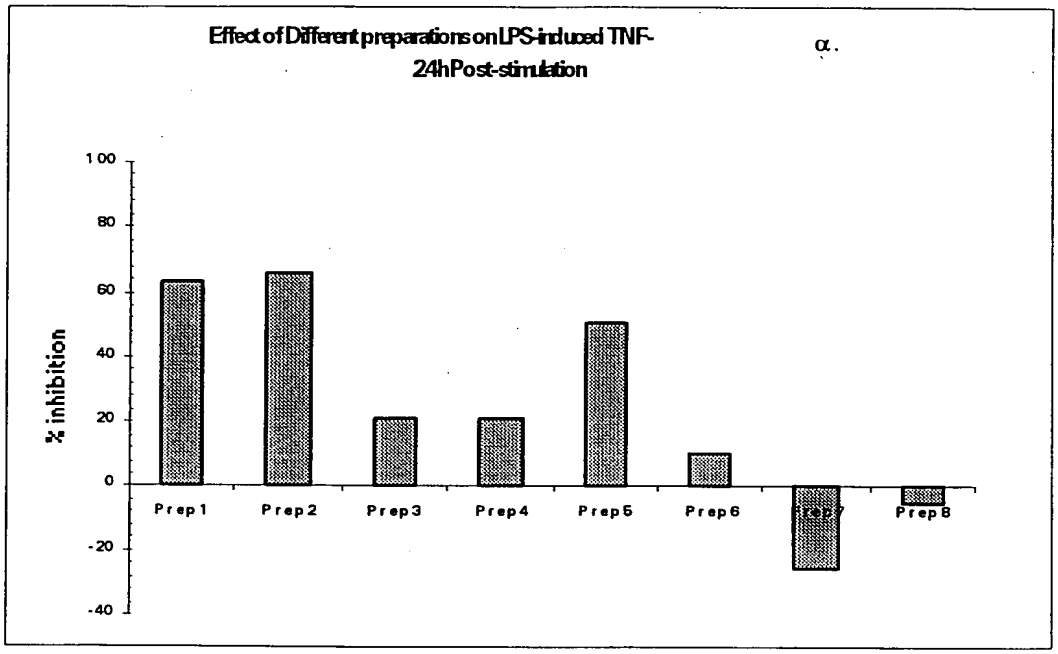
LPS was added to PBMC immediately prior to the addition of cells.
Cells were harvested 48h post-stimulation and the concentration of IL-1α was measured by sandwich ELISA.

Experiment 16

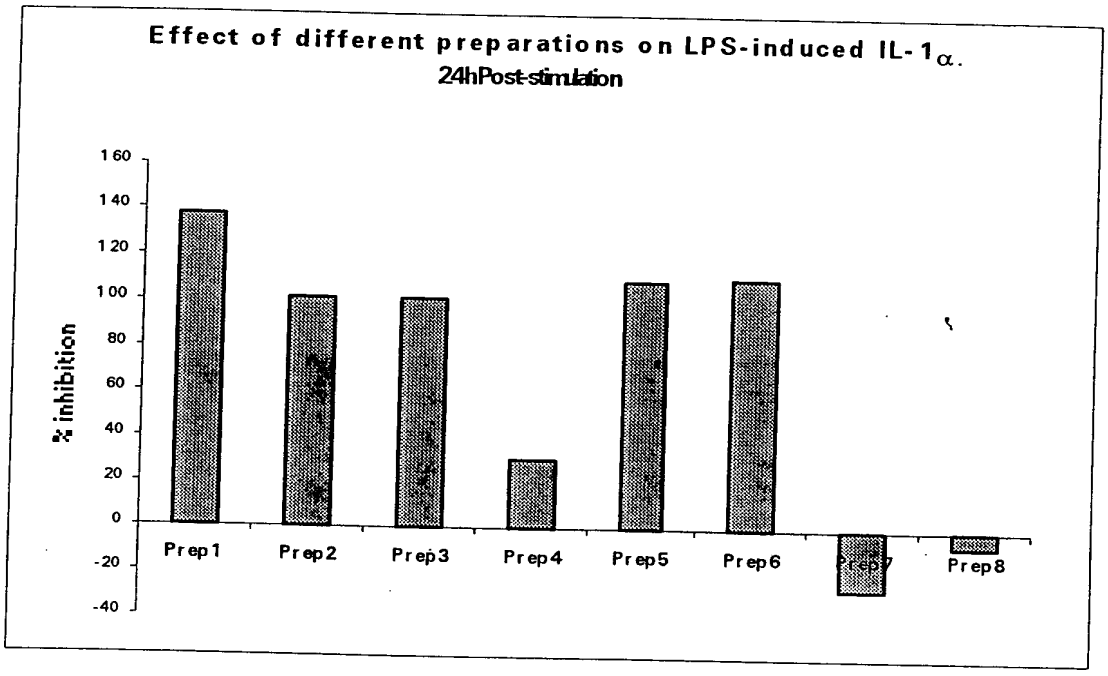
Fig 5



Comparison of biological activities of eight preparations.
In each case 40µl of each preparation was added to PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS.
Preparations 1, 2 and 5 are preparations of Nephronin using slightly different methodology. Preparations 3,4 and 6 are the control preparations respectively.
Preparations 7 and 8 refer to the solvent soluble material after treatment of Na3Citrate with the appropriate acids that was decanted and processed as above.
Cells were harvested 24h post-stimulation and the concentration of TNF-α was measured by sandwich ELISA.
Negative values indicate activation of cells.

Experiment 16

Fig 6



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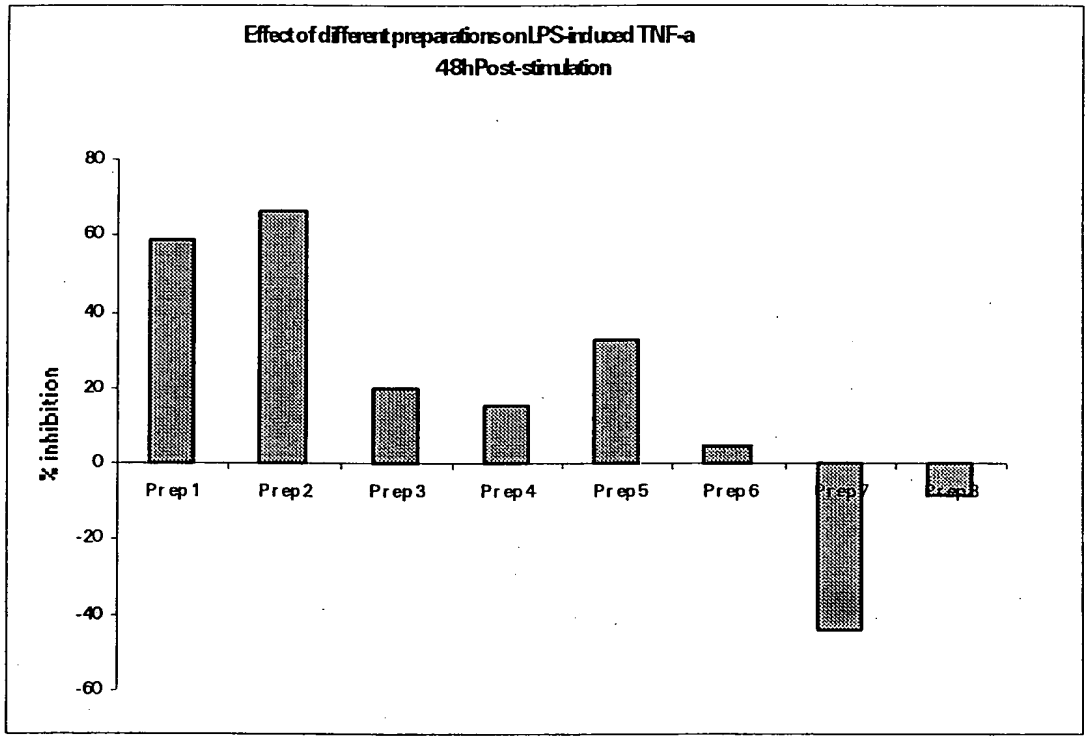
Cells were harvested 24h post-stimulation and the concentration of IL-1α was measured by sandwich ELISA.
Negative values indicate activation of cells.

Experiment 16

Fig 7



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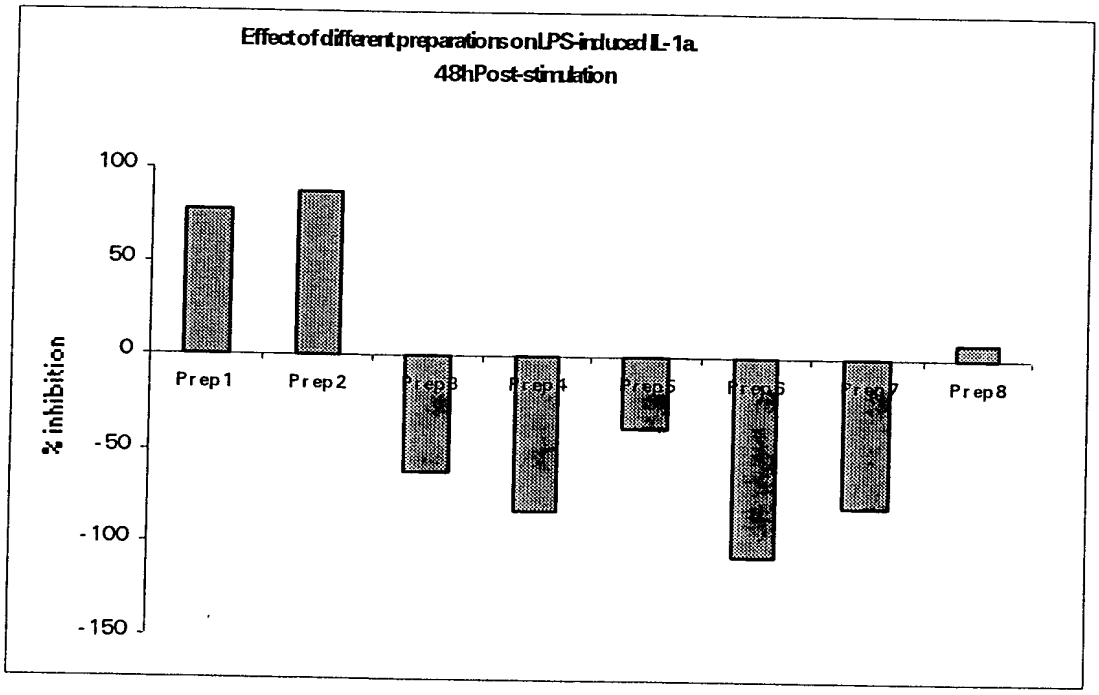
Cells were harvested 48h post-stimulation and the concentration of TNF-α was measured by sandwich ELISA.
Negative values indicate activation of cells.

Experiment 16

Fig 8



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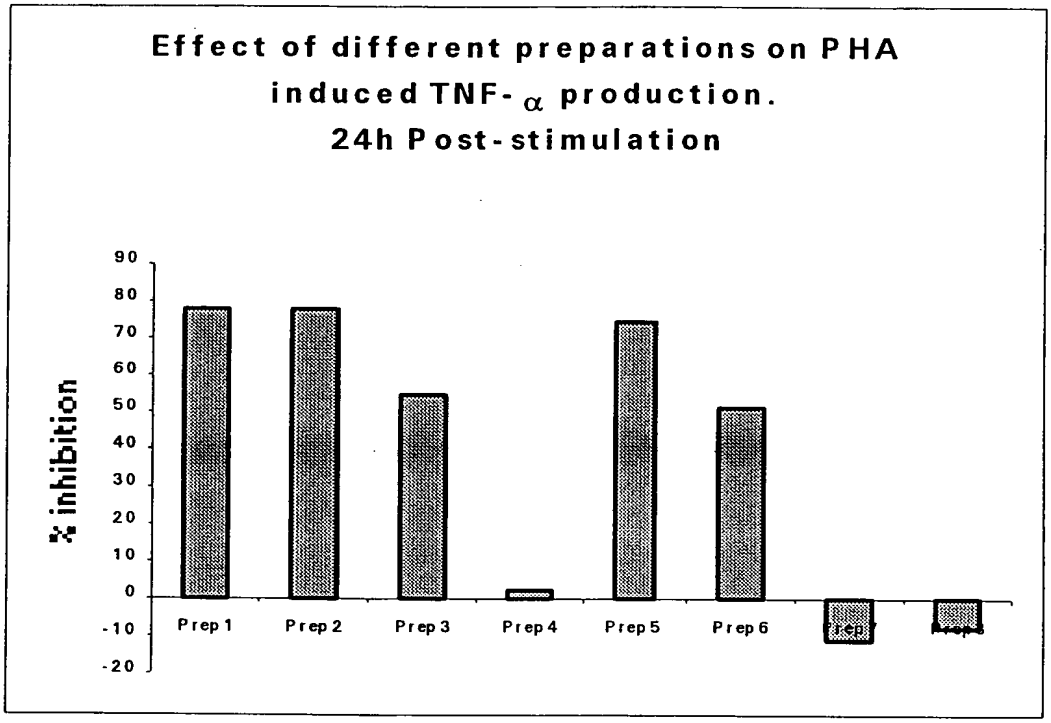
Cells were harvested 48h post-stimulation and the concentration of IL-1α was measured by sandwich ELISA.
Negative values indicate activation of cells.

Experiment 16

Fig 9



Comparison of biological activities of eight preparations.
In each case 40µl of each preparation was added to chambers of 24 well tissue culture plates. To each well, 5µg of PHA was added followed by 1ml to PBMC at $1.5x10^6$ cells/ml.
Preparations 1, 2 and 5 are preparations of Nephronin using slightly different methodology.
Preparations 3,4 and 6 are the control preparations respectively.
Preparations 7 and 8 refer to the solvent soluble material after treatment of Na3Citrate with the appropriate acids that was decanted and processed as above.
Cells were harvested 24h post-stimulation and the concentration of TNF-α was measured by sandwich ELISA.
Negative values indicate activation of cells.

Experiment 16

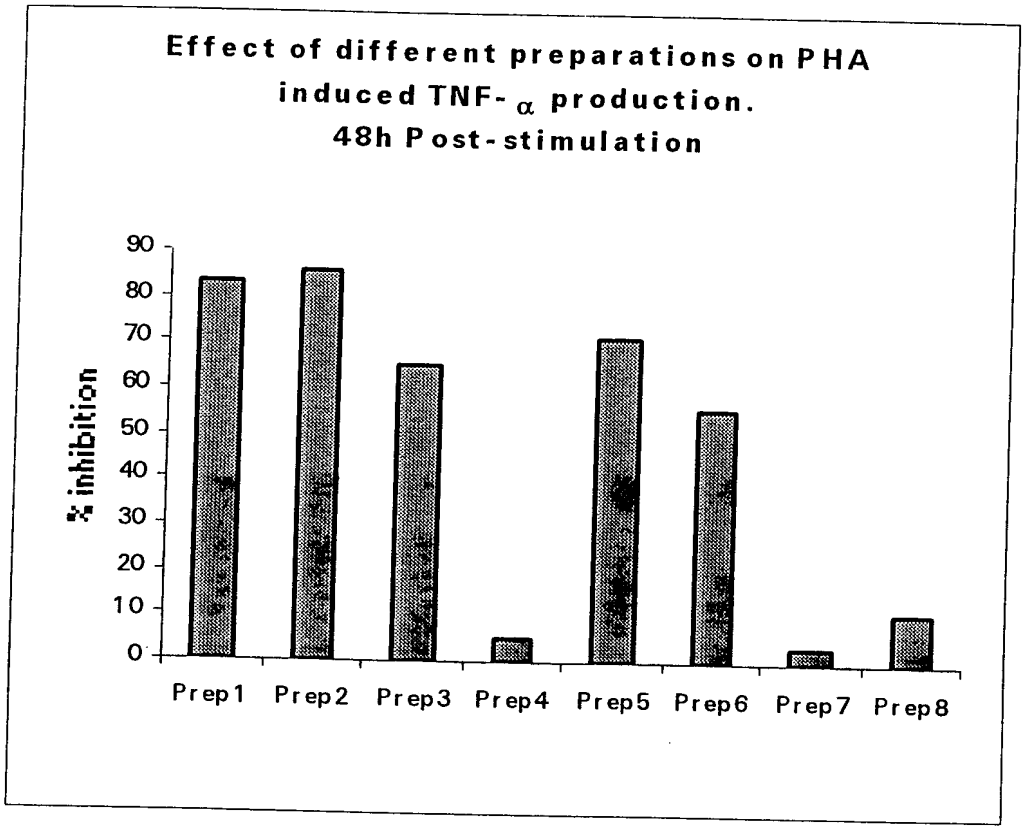


Fig 10

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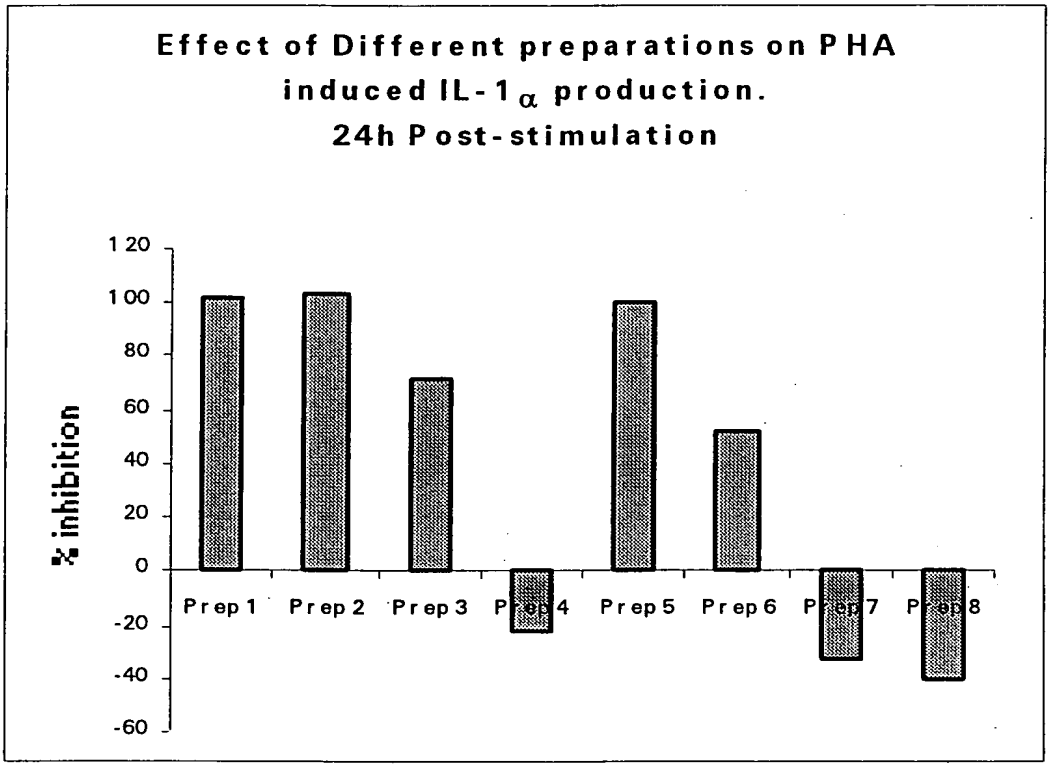
Preparations 7 and 8 refer to the solvent soluble material after treatment of Na3Citrate with the appropriate acids that was decanted and processed as above.
Cells were harvested 48h post-stimulation and the concentration of TNF-α was measured by sandwich ELISA.
Negative values indicate activation of cells.

Experiment 16

Fig 11



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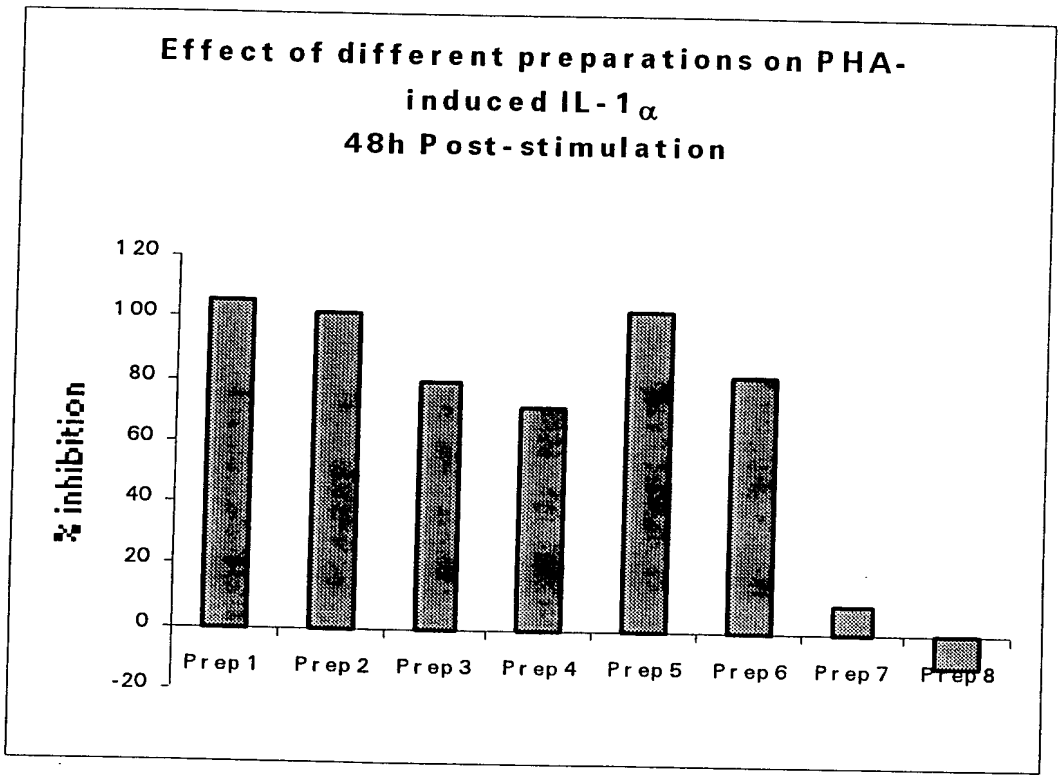
Preparations 7 and 8 refer to the solvent soluble material after treatment of Na3Citrate with the appropriate acids that was decanted and processed as above.
Cells were harvested 24h post-stimulation and the concentration of IL-1α was measured by sandwich ELISA.
Negative values indicate activation of cells.

Experiment 16

Fig 12



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In each case 40µl of each preparation was added to chambers of 24 well tissue culture plates. To each well, 5µg of PHA was added followed by 1ml to PBMC at $1.5x10^6$ cells/ml.
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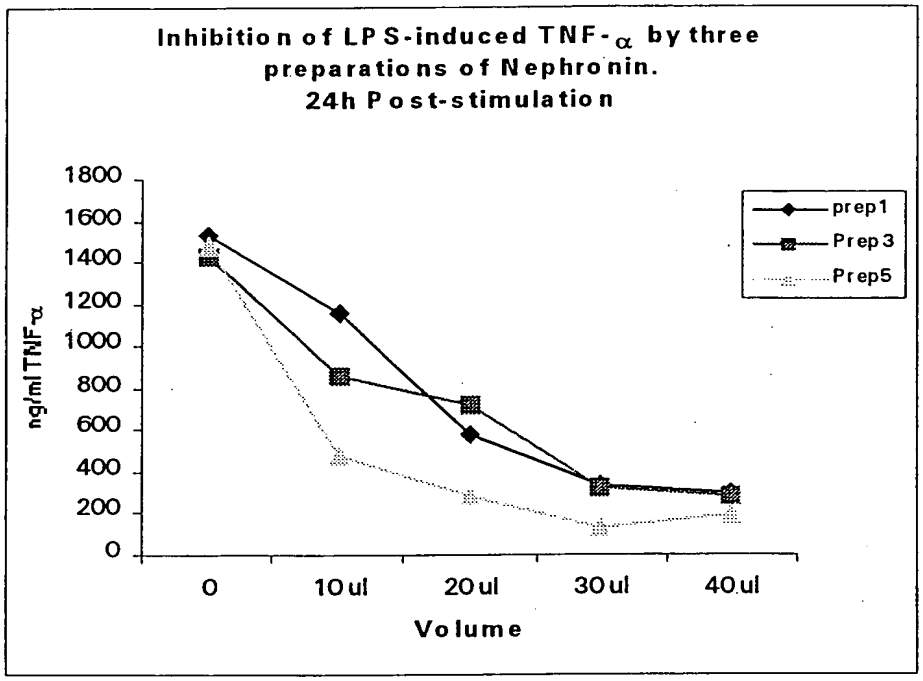
Cells were harvested 48h post-stimulation and the concentration of IL-1α was measured by sandwich ELISA.
Negative values indicate activation of cells.

Experiment 17

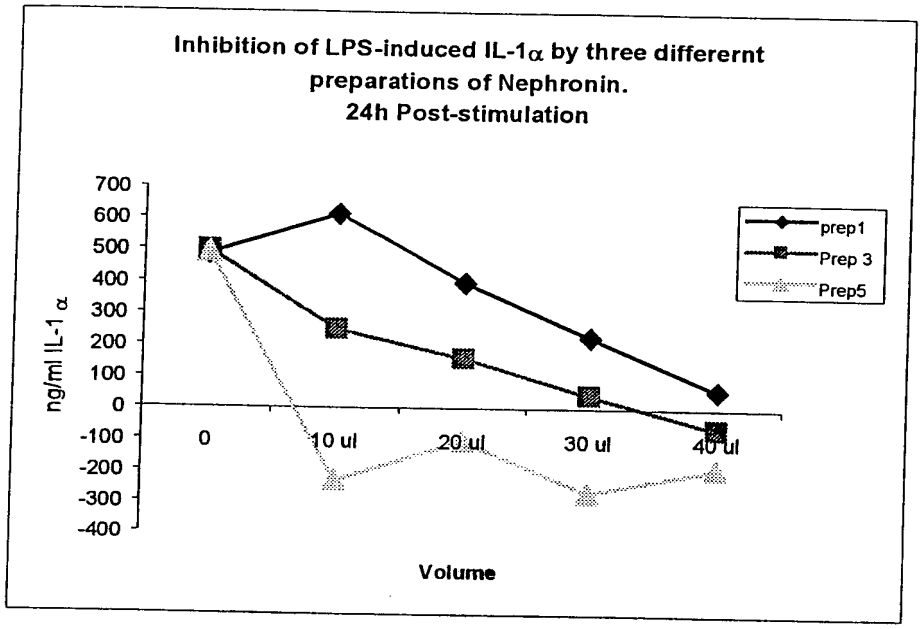
Fig 1



Three preparations where acids of increasing carbon chain length were used to synthesise Nephronin. An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
Prep 1, refers to preparation of Nephronin where acetic acid was used in the synthesis procedure.
In Prep's 3 and 5, refers to Propanoic and Butyric acid respectively in the synthesis procedure, as in Preparation 1.
In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α measured using sandwich ELISA.

Experiment 17

Fig 2



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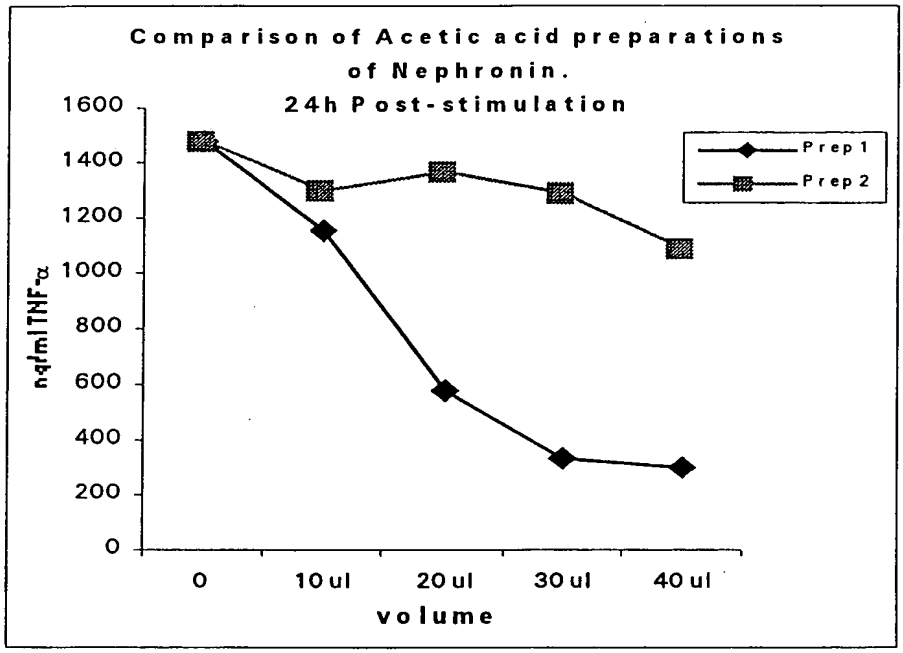
In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α measured using sandwich ELISA.

Experiment 17

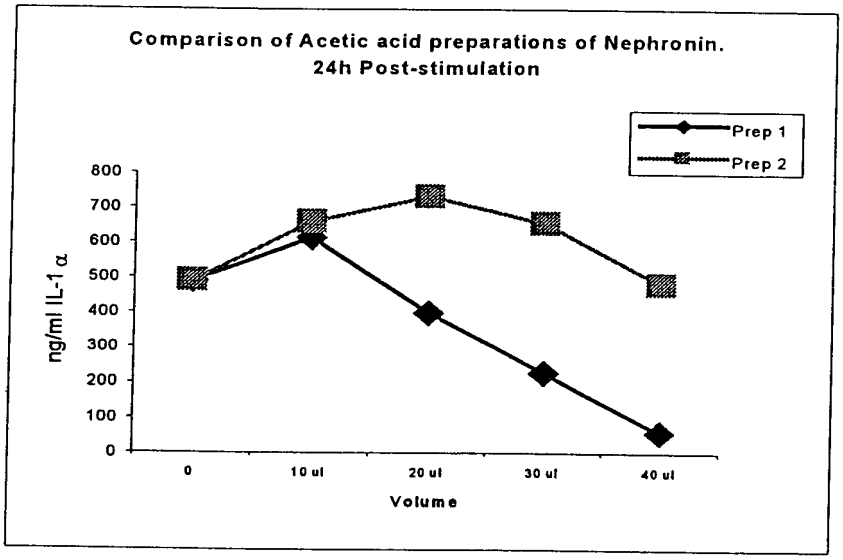
Fig 3



Comparison between preparations of Nephronin, synthesised from the acid soluble and the solid from the treatment of Na3Citrate with acetic acid.
A dose dependent inhibition of LPS-induced TNF-α production, produced by an increasing volume of each preparation.
Prep 1, refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid and the mixture used in the synthesis procedure.
Prep 2, refers to preparation where acetic acid was added to Na3Citrate and after 2h incubation with the citrate, the acid soluble material was used in the synthesis procedure.
In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α measured using sandwich ELISA.

Experiment 17

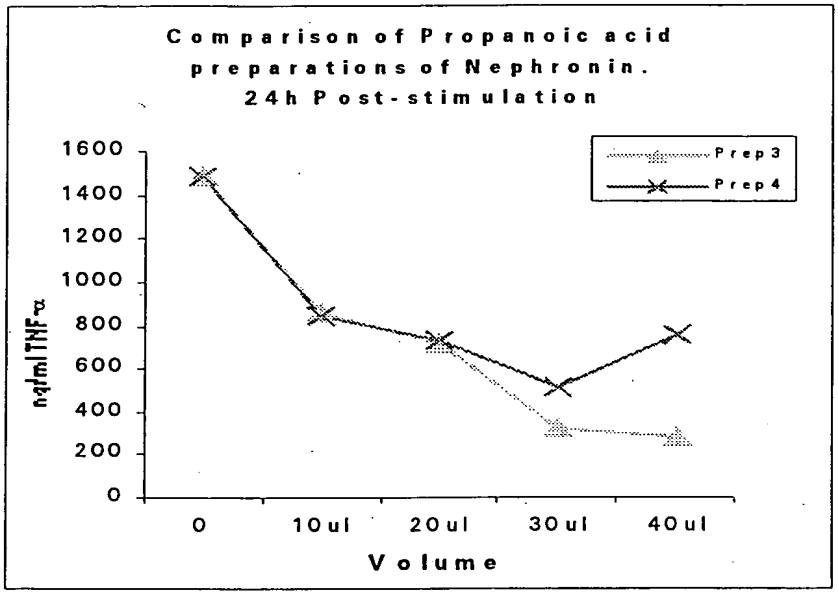
Fig 4



Comparison between preparations of Nephronin, synthesised from the acid soluble and the solid from the treatment of Na3Citrate with acetic acid.
A dose dependent inhibition of LPS-induced IL-1α production, produced by an increasing volume of each preparation.
Prep 1, refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid and the mixture used in the synthesis procedure.
Prep 2, refers to preparation where acetic acid was added to Na3Citrate and after 2h incubation with the citrate, the acid soluble material was used in the synthesis procedure.
In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α measured using sandwich ELISA.

Experiment 17

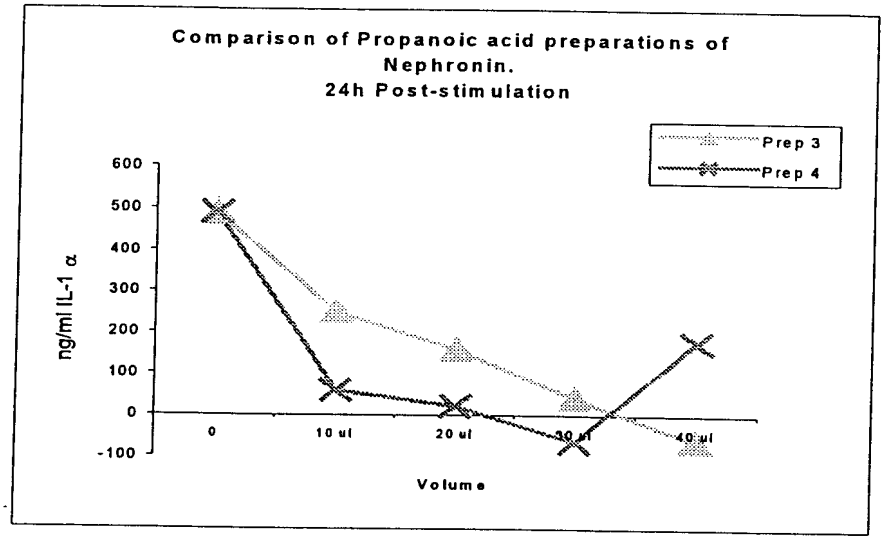
Fig 5



Comparison between preparations of Nephronin, synthesised from the acid soluble and the solid from the treatment of Na3Citrate with propanoic acid.
A dose dependent inhibition of LPS-induced TNF-α production, produced by an increasing volume of each preparation.
Prep 3, refers to preparation of Nephronin where Na3Citrate was first treated with propanoic acid and the mixture used in the synthesis procedure.
Prep 4, refers to preparation where propanoic acid was added to Na3Citrate and after 2h incubation with the citrate, the acid soluble material was used in the synthesis procedure.
In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α measured using sandwich ELISA.

Experiment 17

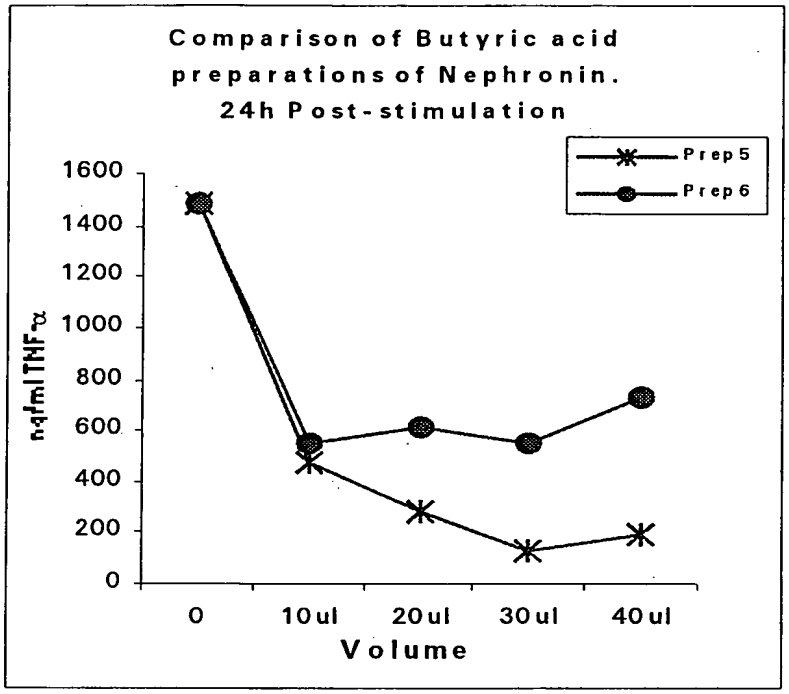
Fig 6



Comparison between preparations of Nephronin, synthesised from the acid soluble and the solid from the treatment of Na3Citrate with propanoic acid.
A dose dependent inhibition of LPS-induced IL-1α production, produced by an increasing volume of each preparation.
Prep 1, refers to preparation of Nephronin where Na3Citrate was first treated with propanoic acid and the mixture used in the synthesis procedure.
Prep 2, refers to preparation where propanoic acid was added to Na3Citrate and after 2h incubation with the citrate, the acid soluble material was used in the synthesis procedure.
In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α measured using sandwich ELISA.

Experiment 17

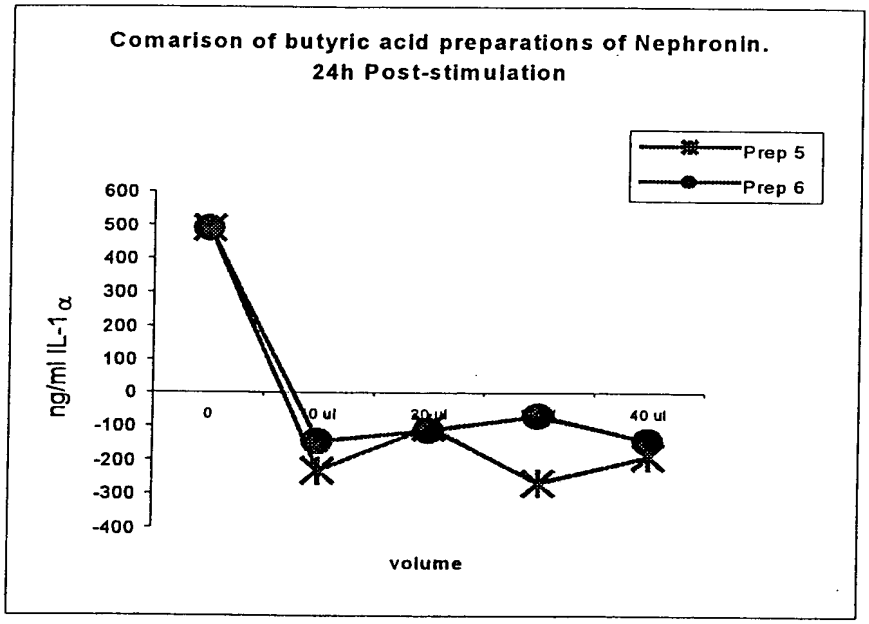
Fig 7



Comparison between preparations of Nephronin, synthesised from the acid soluble and the solid from the treatment of Na3Citrate with butyric acid.
A dose dependent inhibition of LPS-induced TNF-α production, produced by an increasing volume of each preparation.
Prep 5, refers to preparation of Nephronin where Na3Citrate was first treated with butyric acid and the mixture used in the synthesis procedure.
Prep 6, refers to preparation where butyric acid was added to Na3Citrate and after 2h incubation with the citrate, the acid soluble material was used in the synthesis procedure.
In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α measured using sandwich ELISA.

Experiment 17

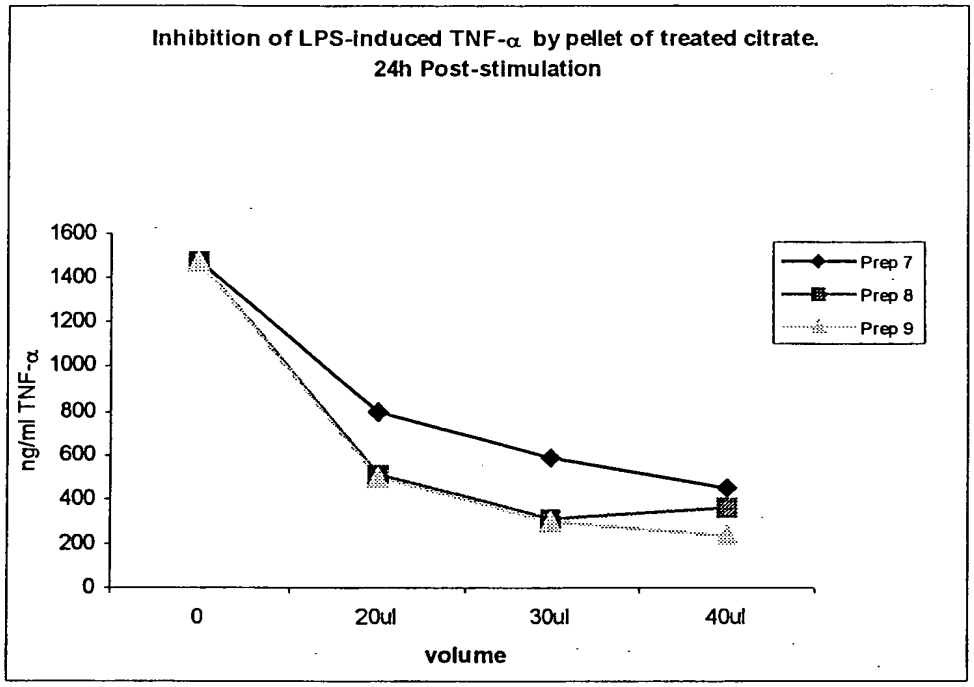
Fig 8



Comparison between preparations of Nephronin, synthesised from the acid soluble and the solid from the treatment of Na3Citrate with butyric acid.
A dose dependent inhibition of LPS-induced IL-1α production, produced by an increasing volume of each preparation.
Prep 5, refers to preparation of Nephronin where Na3Citrate was first treated with butyric acid and the mixture used in the synthesis procedure.
Prep 6, refers to preparation where butyric acid was added to Na3Citrate and after 2h incubation with the citrate, the acid soluble material was used in the synthesis procedure.
In each case, the appropriate volume of the preparations was added to 24 well tissue culture plate. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α measured using sandwich ELISA.

Experiment 17

Fig 9



Comparison between Na3Citrate treated first with the appropriate acid. After removal of the acid soluble material, the resulting material was then used in the synthesis procedure. A dose dependent inhibition of LPS-induced TNF-α production, produced by an increasing volume of each preparation.

Prep 7, refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid and after removing the acid, the remaining material used in the synthesis procedure.

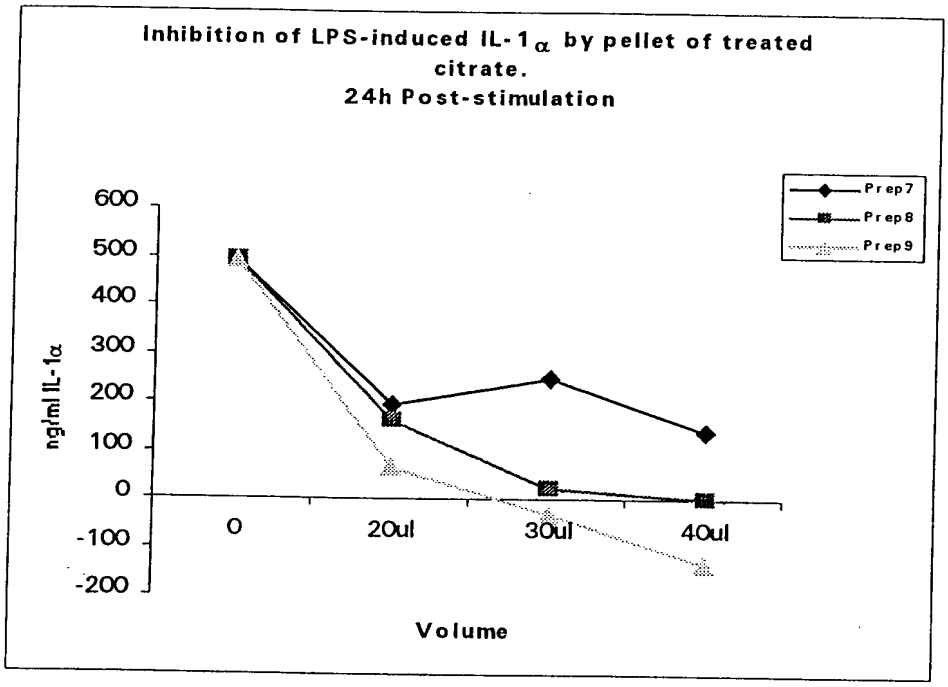
Prep 8, refers to preparation of Nephronin where Na3Citrate was first treated with propanoic acid and after removing the acid, the remaining material used in the synthesis procedure.

Prep 9, refers to preparation of Nephronin where Na3Citrate was first treated with butyric acid and after removing the acid, the remaining material used in the synthesis procedure.

In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α measured using sandwich ELISA

Experiment 17

Fig 10



Comparison between Na3Citrate treated first with the appropriate acid. After removal of the acid soluble material, the resulting material was then used in the synthesis procedure. A dose dependent inhibition of LPS-induced IL-1α production, produced by an increasing volume of each preparation.

Prep 7, refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid and after removing the acid, the remaining material used in the synthesis procedure.

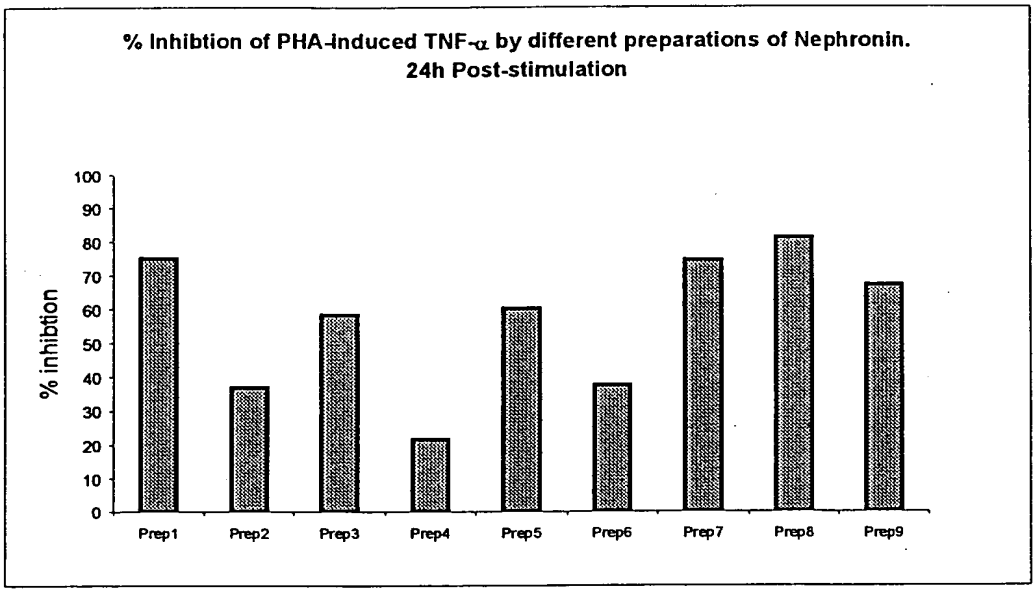
Prep 8, refers to preparation of Nephronin where Na3Citrate was first treated with propanoic acid and after removing the acid, the remaining material used in the synthesis procedure.

Prep 9, refers to preparation of Nephronin where Na3Citrate was first treated with butyric acid and after removing the acid, the remaining material used in the synthesis procedure.

In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α measured using sandwich ELISA.

Experiment 17

Fig 11



Comparison of nine preparations using acetic acid, propanoic acid and butyric acid in the synthesis procedure. Minor modifications in the treatment of citrate were introduced and the synthesised material tested for their ability to inhibit PHA-induced TNF-α production.

Preps 1, 3 and 5 refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid, propanoic acid, and butyric acid respectively, and the mixture used in the synthesis procedure.

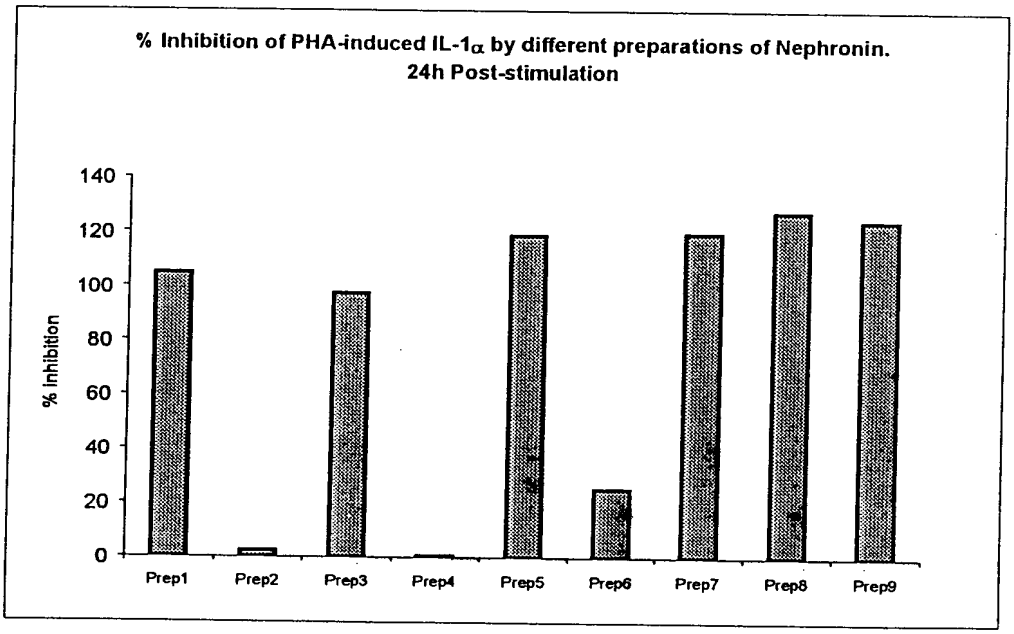
Preps 2, 4 and 6 refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid, propanoic acid and butyric acid respectively, and the resulting acid soluble material was decanted and used in the synthesis procedure.

Preps 7, 8 and 9 refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid, propanoic acid and butyric acid respectively, and after removing the acid soluble material, the solids were used in the synthesis procedure.

In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α measured using sandwich ELISA.

Experiment 17

Fig 12



Comparison of nine preparations using acetic acid, propanoic acid and butyric acid in the synthesis procedure. Minor modifications in the treatment of citrate were introduced and the synthesised material tested for their ability to inhibit PHA-induced IL-1α production.

Preps 1, 3 and 5 refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid, propanoic acid, and butyric acid respectively, and the mixture used in the synthesis procedure.

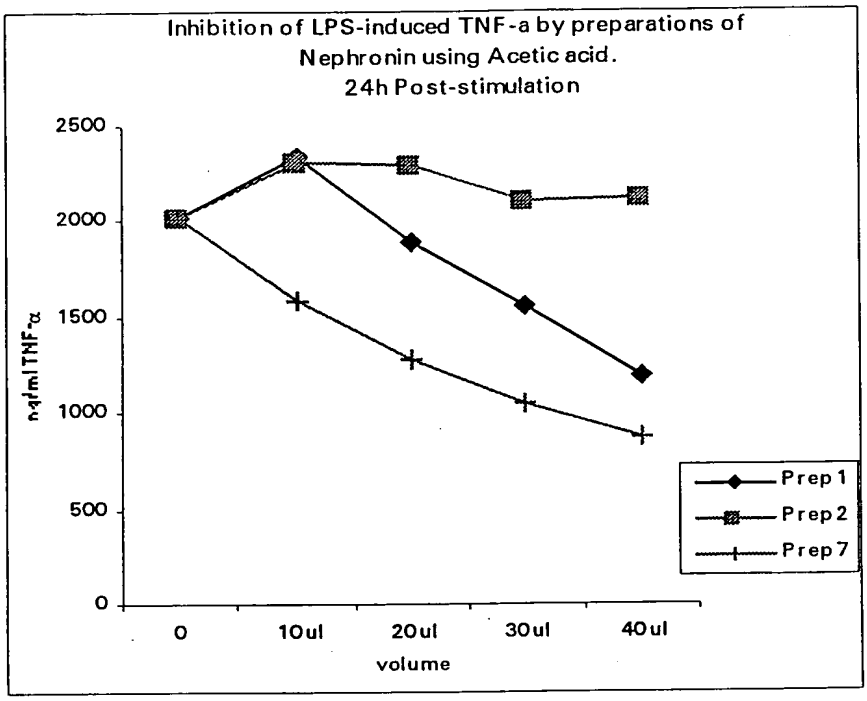
Preps 2, 4 and 6 refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid, propanoic acid and butyric acid respectively, and the resulting acid soluble material was decanted and used in the synthesis procedure.

Preps 7, 8 and 9 refers to preparation of Nephronin where Na3Citrate was first treated with acetic acid, propanoic acid and butyric acid respectively, and after removing the acid soluble material, the solids were used in the synthesis procedure.

In each case, the appropriate volume of the preparations was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS were then added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α measured using sandwich ELISA.

Experiment 17R

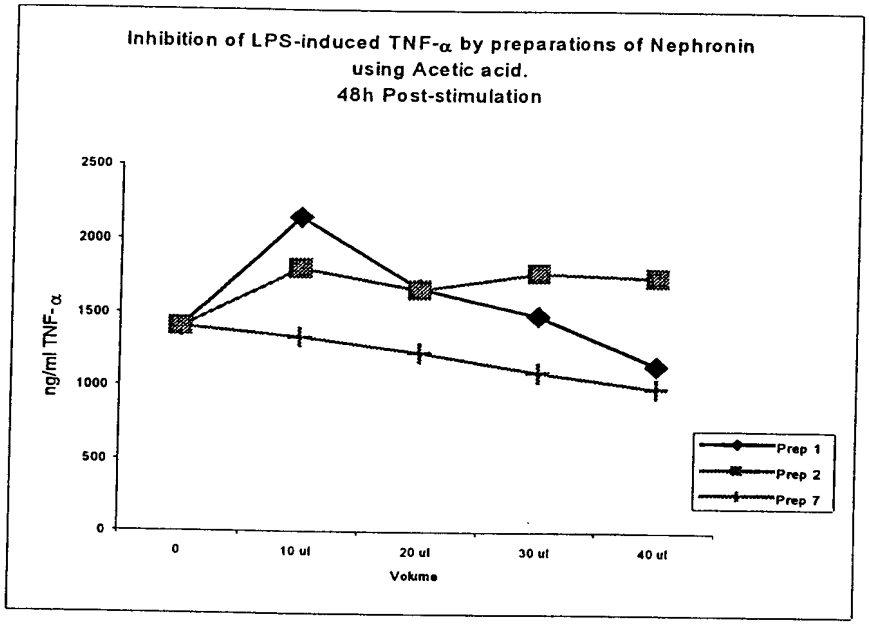
Fig 1



Dose dependent inhibition of LPS-induced TNF-α production, by three preparations of Nephronin using acetic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with acetic acid and the mixture used in the synthesis.
Prep 2 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with acetic acid, the acid soluble material was used in the synthesis procedure.
Prep 7 refers to preparation of Nephronin, where after pre-treatment of Na3Citrate with acetic acid and removal of the acid soluble material, the remaining solid was used in the synthesis procedure.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 17R

Fig 2



Dose dependent inhibition of LPS-induced TNF-α production, by three preparations of Nephronin using acetic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with acetic acid and the mixture used in the synthesis.
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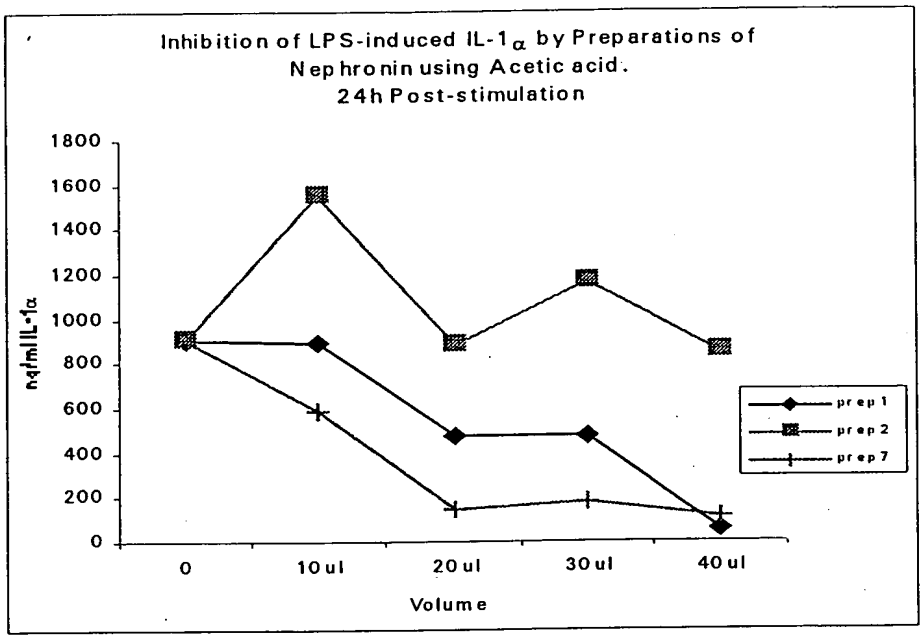
Experiment 17R

Fig 3



Dose dependent inhibition of LPS-induced IL-1α production, by three preparations of Nephronin using acetic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with acetic acid and the mixture used in the synthesis.
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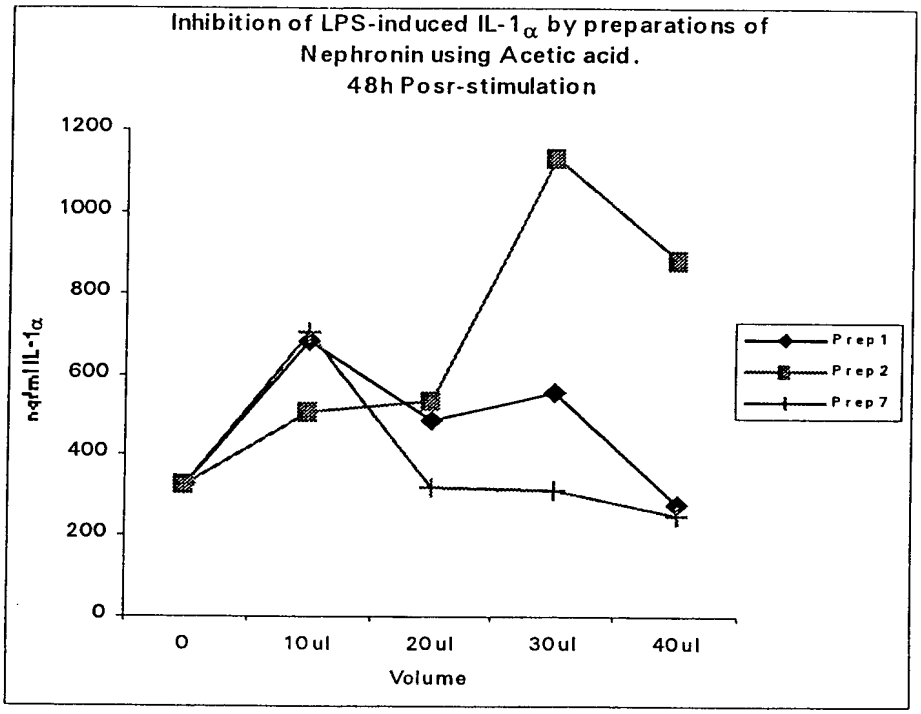
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 17R

Fig 4



Dose dependent inhibition of LPS-induced IL-1α production, by three preparations of Nephronin using acetic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with acetic acid and the mixture used in the synthesis.
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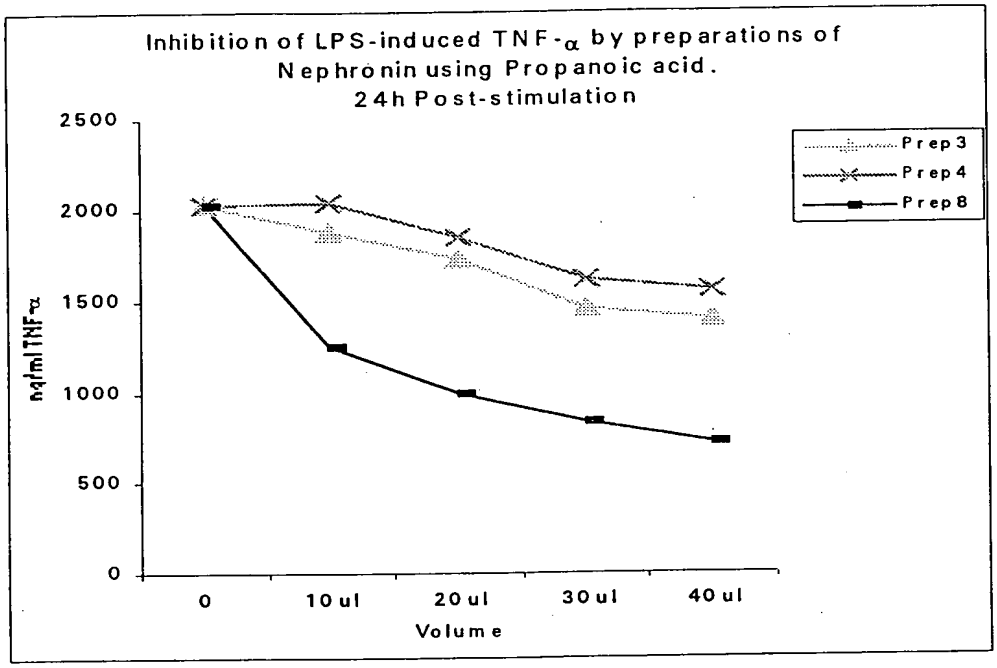
Experiment 17R

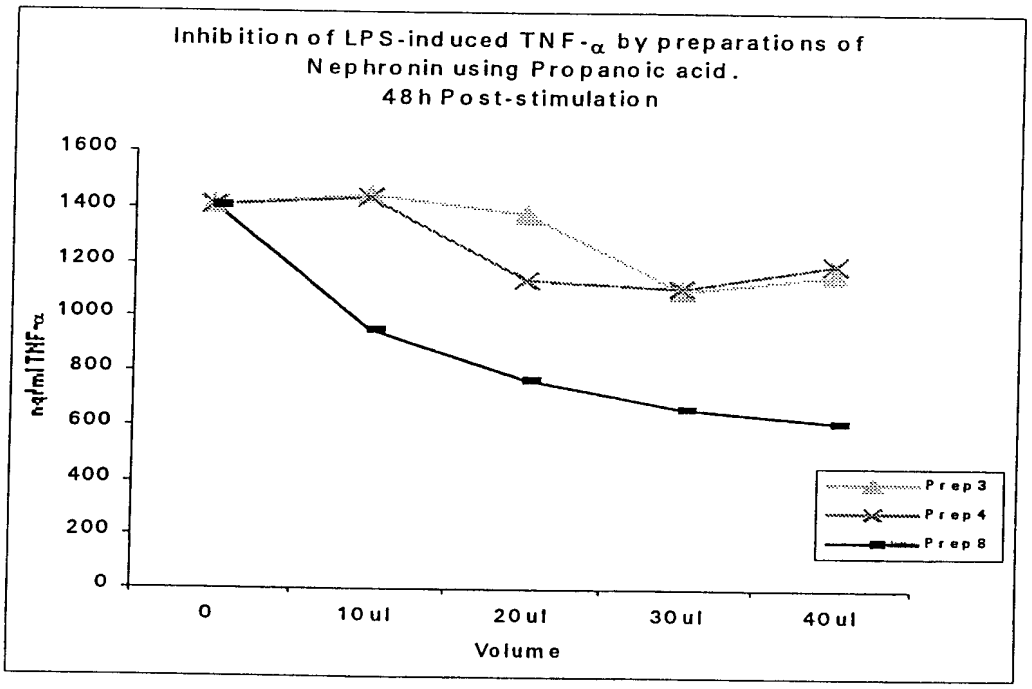
Fig 5



Dose dependent inhibition of LPS-induced TNF-α production, by three preparations of Nephronin using Propanoic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with Propanoic acid and the mixture used in the synthesis.
Prep 2 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with Propanoic acid, the acid soluble material was used in the synthesis procedure.
Prep 7 refers to preparation of Nephronin, where after pre-treatment of Na3Citrate with Propanoic acid and removal of the acid soluble material, the remaining solid was used in the synthesis procedure.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 17R

Fig 6



Dose dependent inhibition of LPS-induced TNF-α production, by three preparations of Nephronin using Propanoic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with Propanoic acid and the mixture used in the synthesis.
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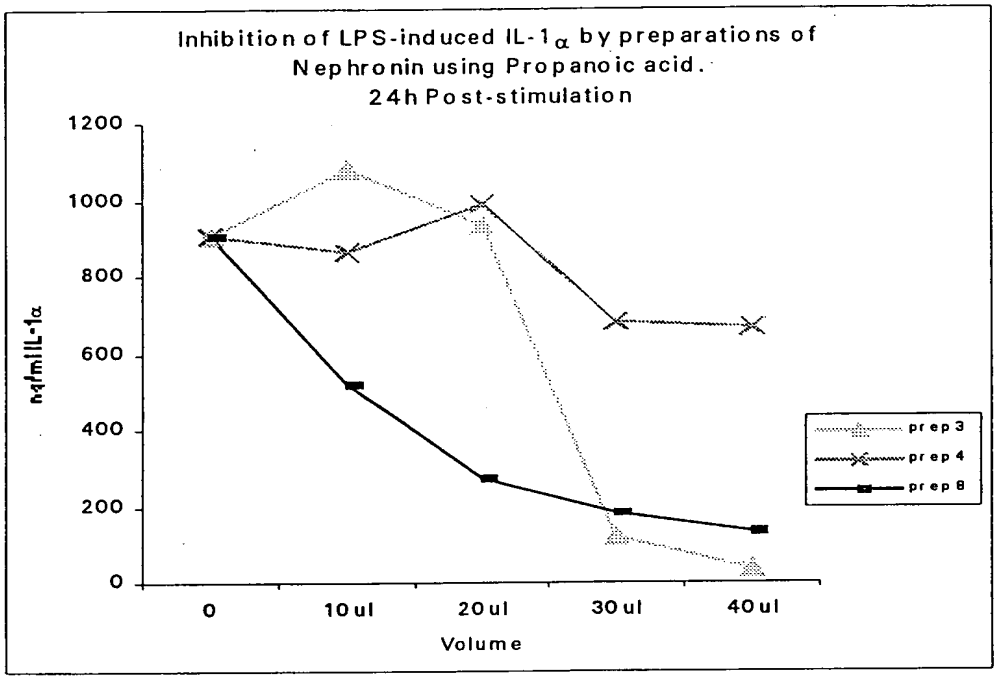
Experiment 17R

Fig 7



Dose dependent inhibition of LPS-induced IL-1α production, by three preparations of Nephronin using Propanoic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with Propanoic acid and the mixture used in the synthesis.
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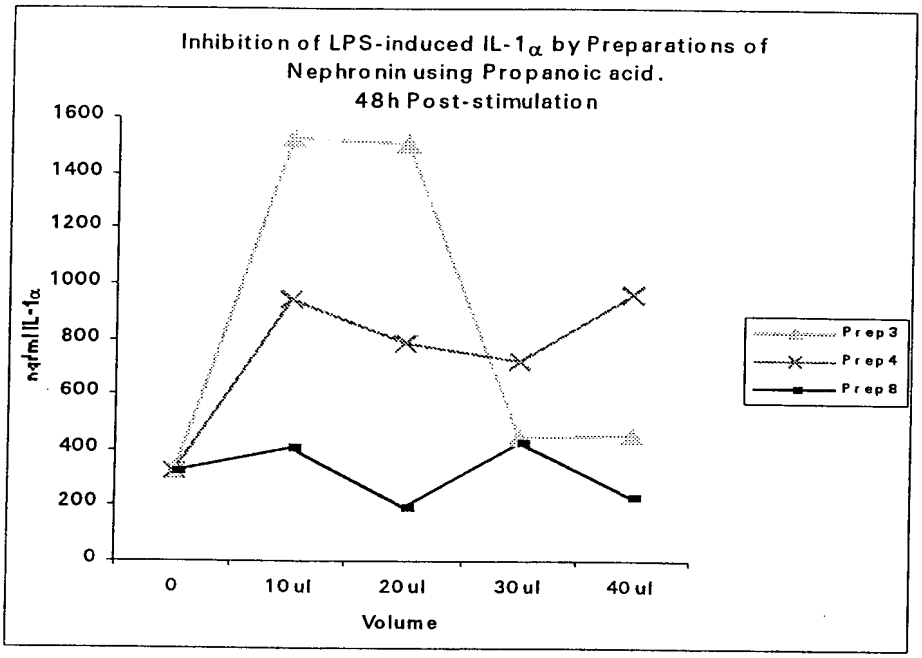
Experiment 17R

Fig 8



Dose dependent inhibition of LPS-induced IL-1α production, by three preparations of Nephronin using Propanoic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with Propanoic acid and the mixture used in the synthesis.
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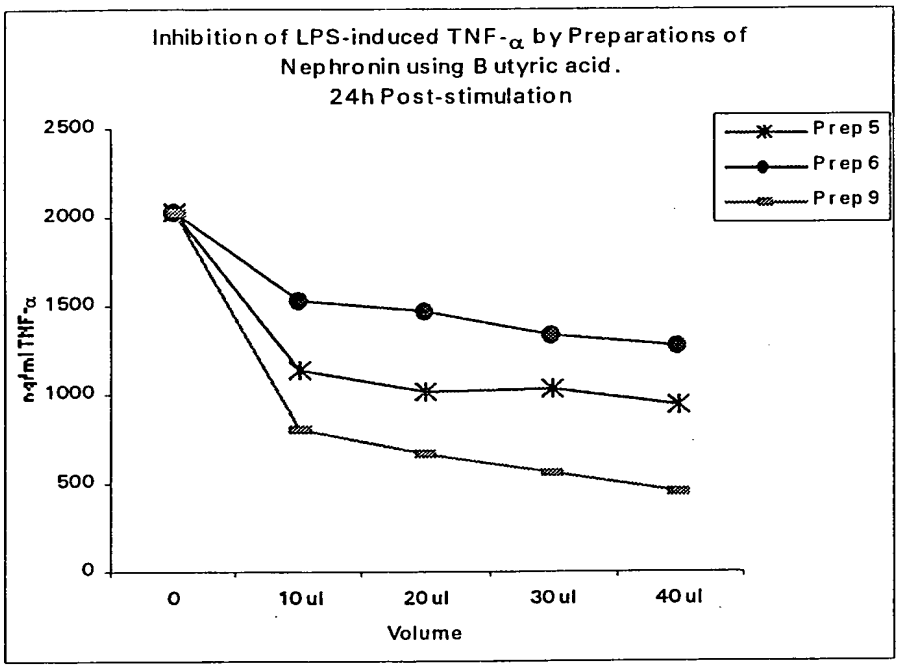
Experiment 17R

Fig 9



Dose dependent inhibition of LPS-induced TNF-α production, by three preparations of Nephronin using Butyric acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with Butyric acid and the mixture used in the synthesis.
Prep 2 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with butyric acid, the acid soluble material was used in the synthesis procedure.
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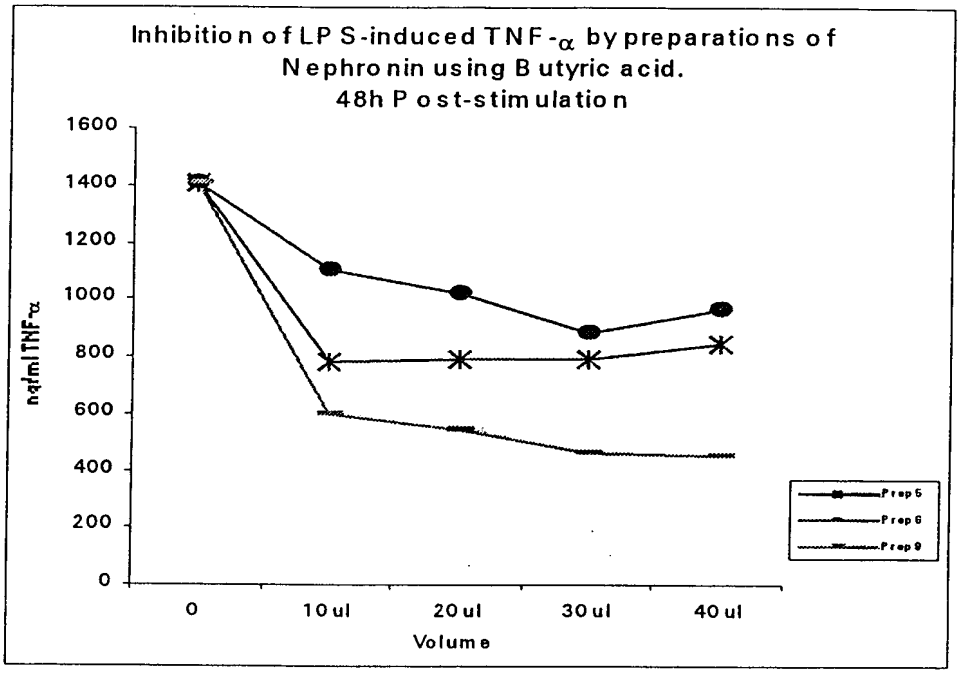
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 17R

Fig 10



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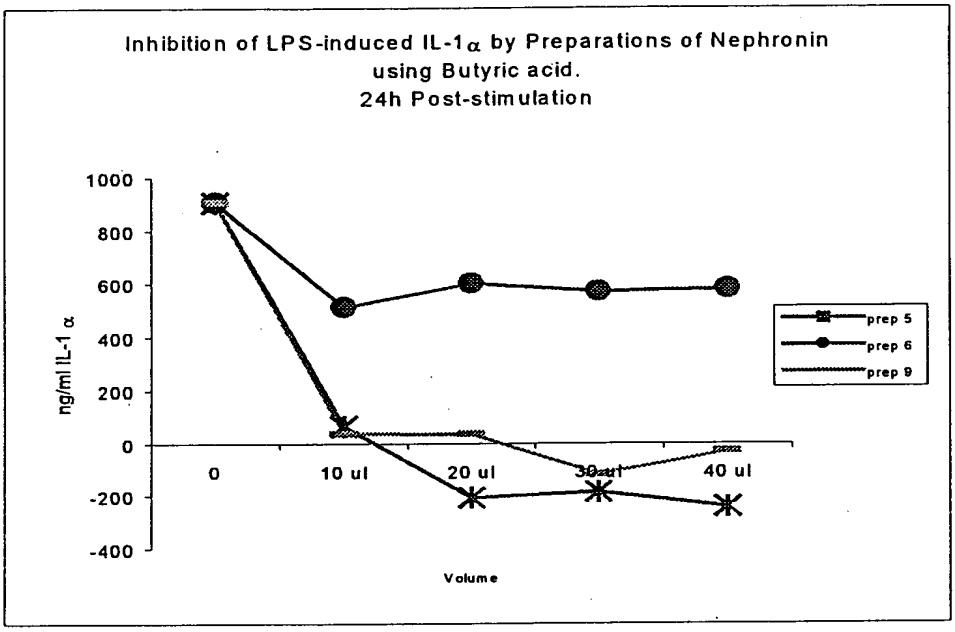
Experiment 17R

Fig 11



Dose dependent inhibition of LPS-induced IL-1α production, by three preparations of Nephronin using Butyric acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with Butyric acid and the mixture used in the synthesis.
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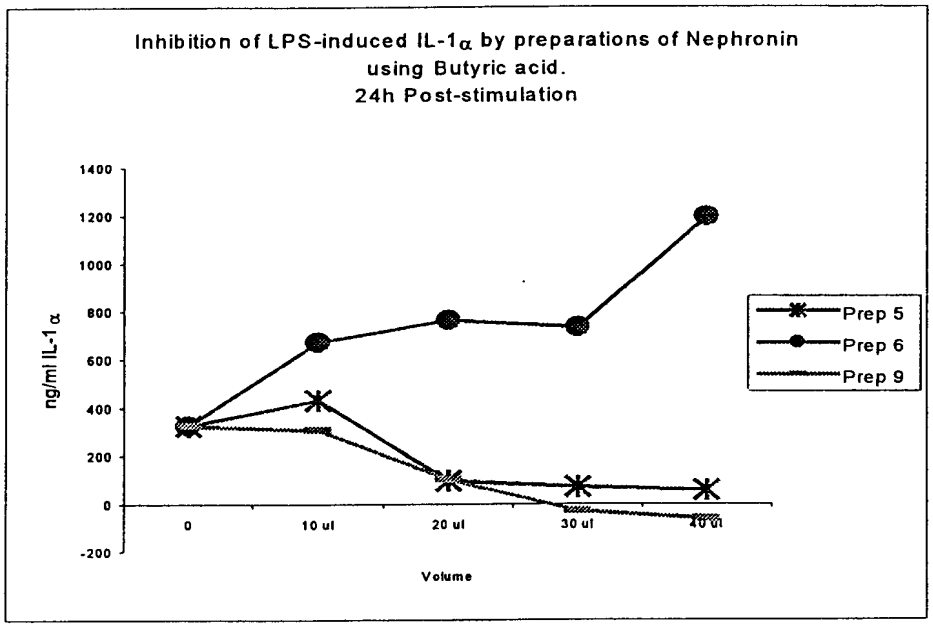
Experiment 17R

Fig 12



Dose dependent inhibition of LPS-induced IL-1α production, by three preparations of Nephronin using Butyric acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with Butyric acid and the mixture used in the synthesis.
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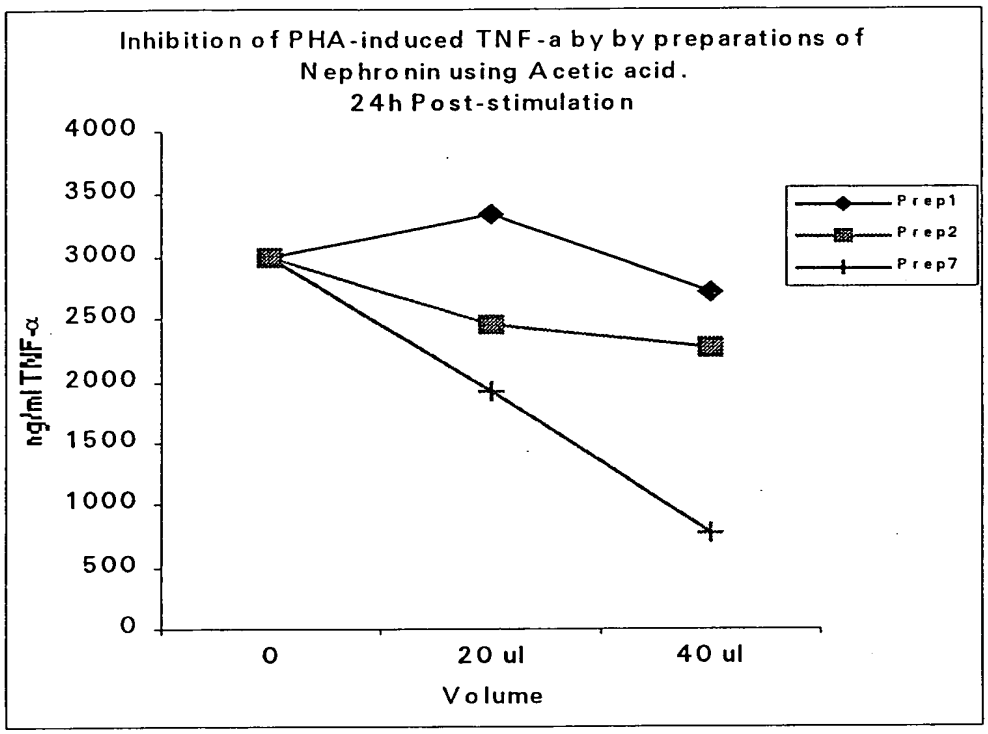
Experiment 17R

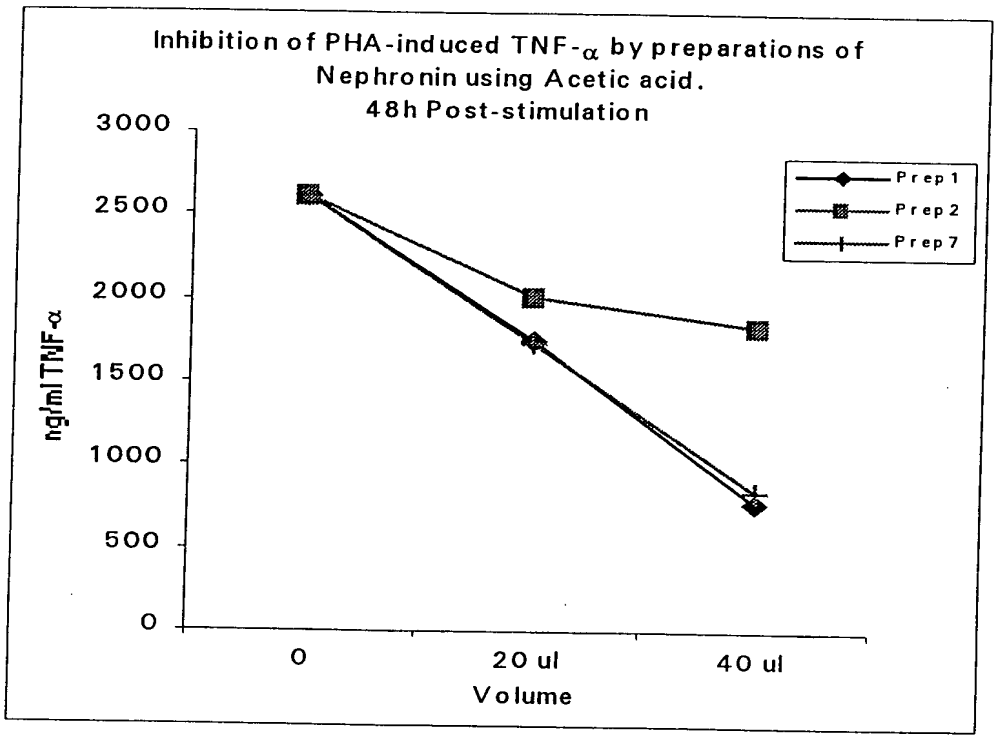
Fig 13



Dose dependent inhibition of PHA-induced TNF-α production by three preparations of Nephronin using acetic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with acetic acid and the mixture used in the synthesis.
Prep 2 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with acetic acid, the acid soluble material was used in the synthesis procedure.
Prep 7 refers to preparation of Nephronin, where after pre-treatment of Na3Citrate with acetic acid and removal of the acid soluble material, the remaining solid was used in the synthesis procedure.
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added and then 1ml of PBMC at $1.5x10^6$ cells/ml was added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 17R

Fig 14



Dose dependent inhibition of PHA-induced TNF-α production by three preparations of Nephronin using acetic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with acetic acid and the mixture used in the synthesis.
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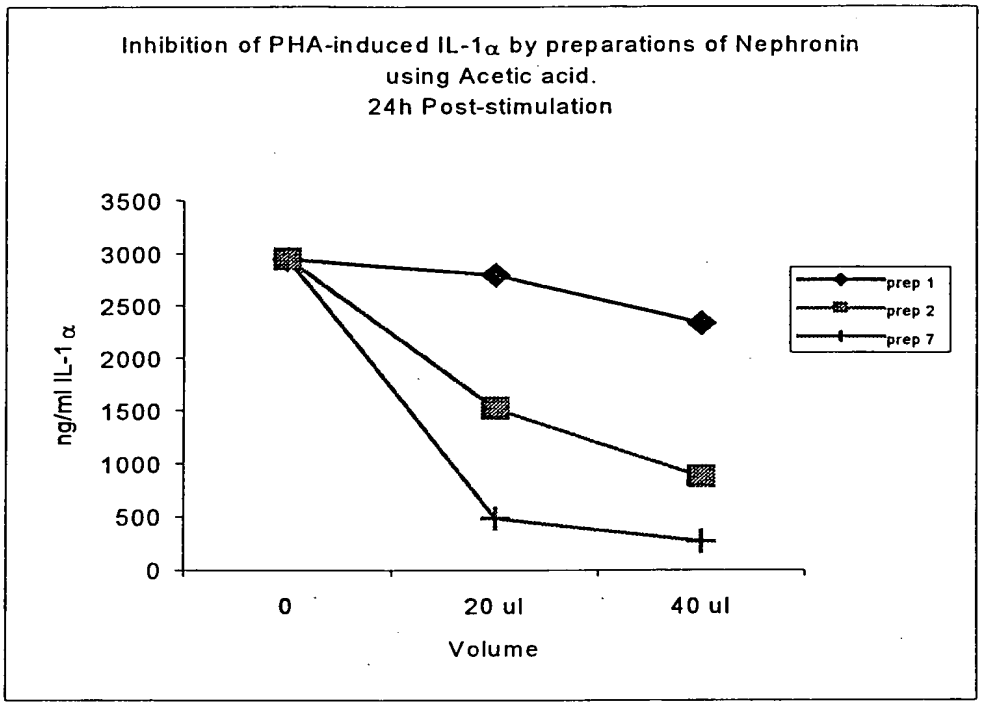
Experiment 17R

Fig 15



Dose dependent inhibition of PHA-induced IL-1α production by three preparations of Nephronin using acetic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with acetic acid and the mixture used in the synthesis.
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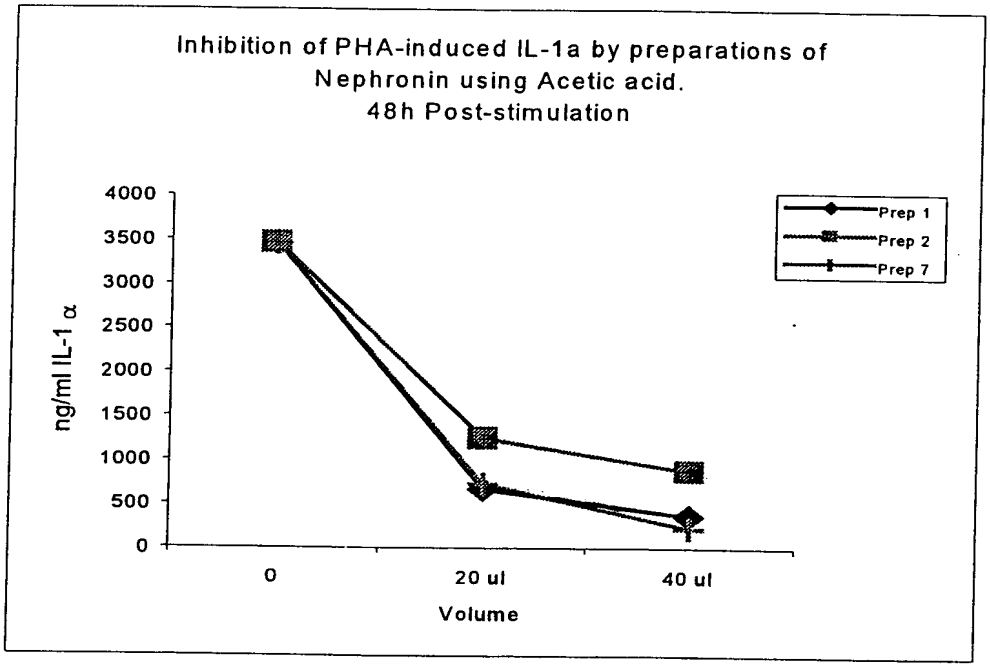
Experiment 17R

Fig 16



Dose dependent inhibition of PHA-induced IL-1α production by three preparations of Nephronin using acetic acid in the synthesis procedure.
Prep 1 refers to preparation of Nephronin, by pre-treating Na3Citrate with acetic acid and the mixture used in the synthesis.
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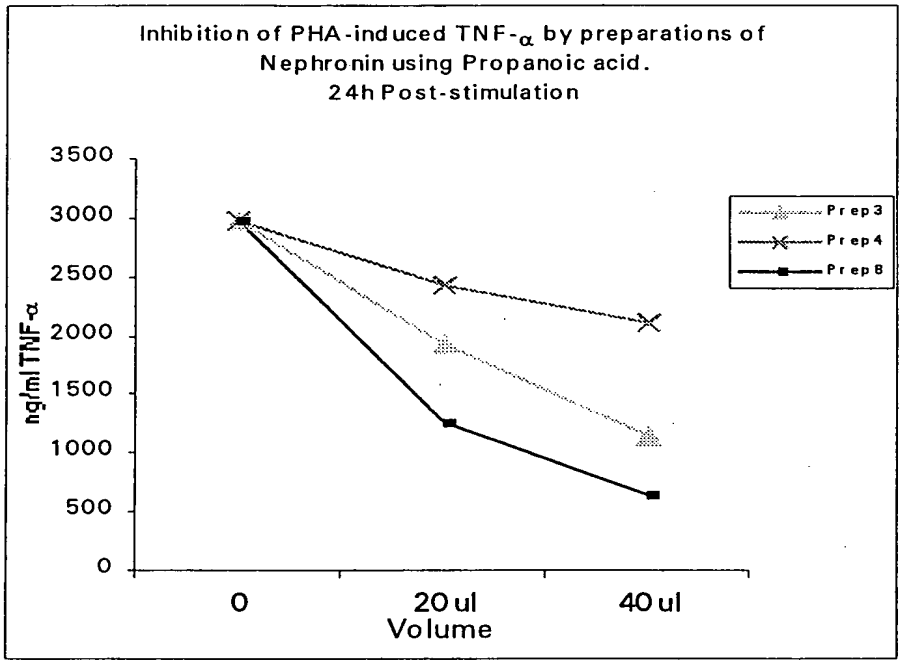
Experiment 17R

Fig 17



Dose dependent inhibition of PHA-induced TNF-α production by three preparations of Nephronin using Propanoic acid in the synthesis procedure.
Prep 3 refers to preparation of Nephronin, by pre-treating Na3Citrate with Propanoic acid and the mixture used in the synthesis.
Prep 4 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with Propanoic acid, the acid soluble material was used in the synthesis procedure.
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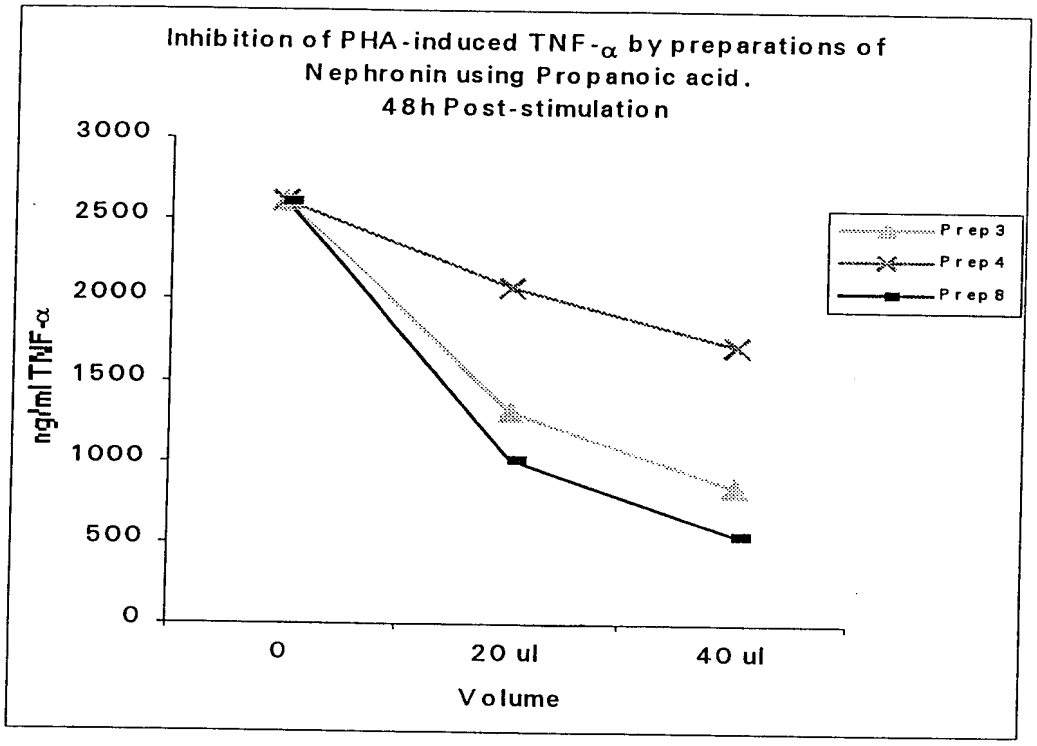
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added and then 1ml of PBMC at 1.5×10^6 cells/ml was added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 17R

Fig 18



Dose dependent inhibition of PHA-induced TNF-α production by three preparations of Nephronin using Propanoic acid in the synthesis procedure.
Prep 3 refers to preparation of Nephronin, by pre-treating Na3Citrate with Propanoic acid and the mixture used in the synthesis.
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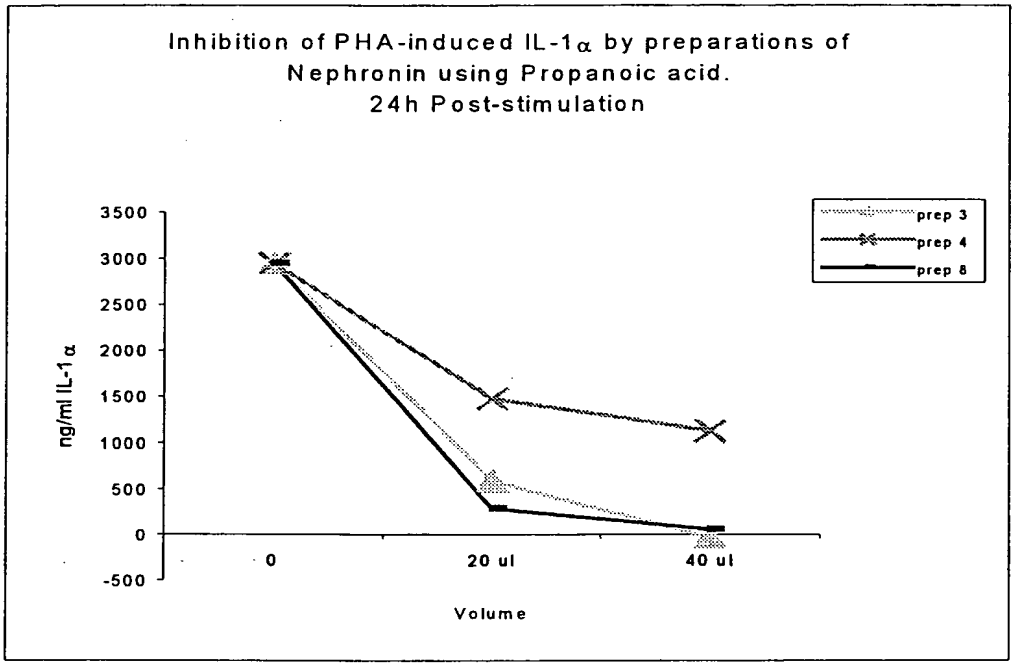
Experiment 17R

Fig 19



Dose dependent inhibition of PHA-induced IL-1α production by three preparations of Nephronin using Propanoic acid in the synthesis procedure.
Prep 3 refers to preparation of Nephronin, by pre-treating Na3Citrate with Propanoic acid and the mixture used in the synthesis.
Prep 4 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with Propanoic acid, the acid soluble material was used in the synthesis procedure.
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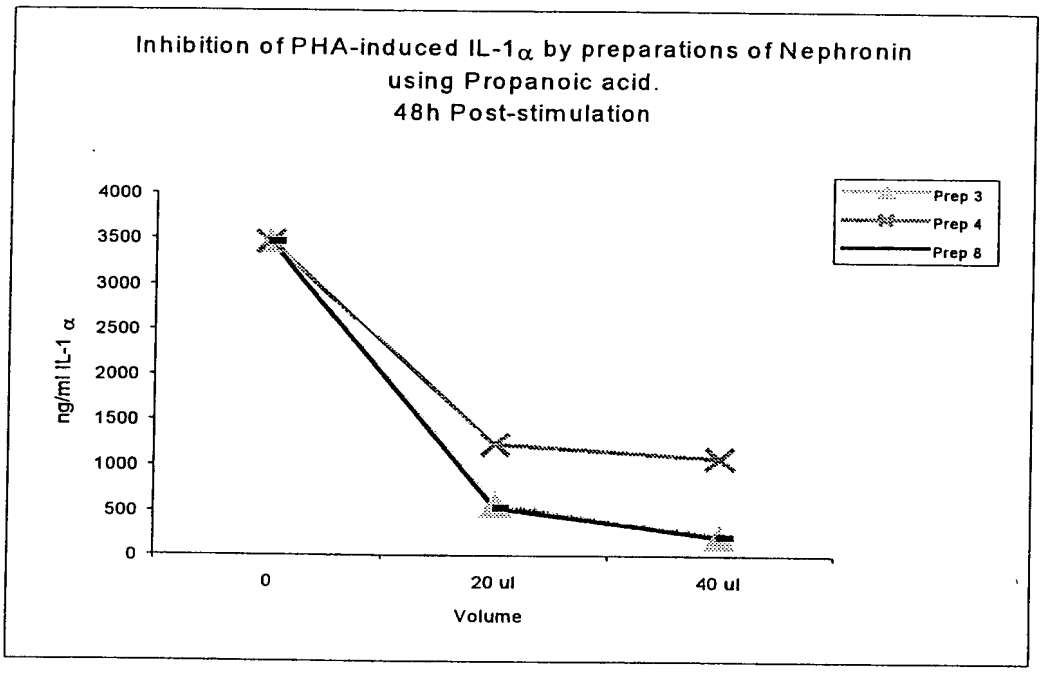
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added and then 1ml of PBMC at $1.5x10^6$ cells/ml was added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 17R

Fig 20



Dose dependent inhibition of PHA-induced IL-1α production by three preparations of Nephronin using Propanoic acid in the synthesis procedure.
Prep 3 refers to preparation of Nephronin, by pre-treating Na3Citrate with Propanoic acid and the mixture used in the synthesis.
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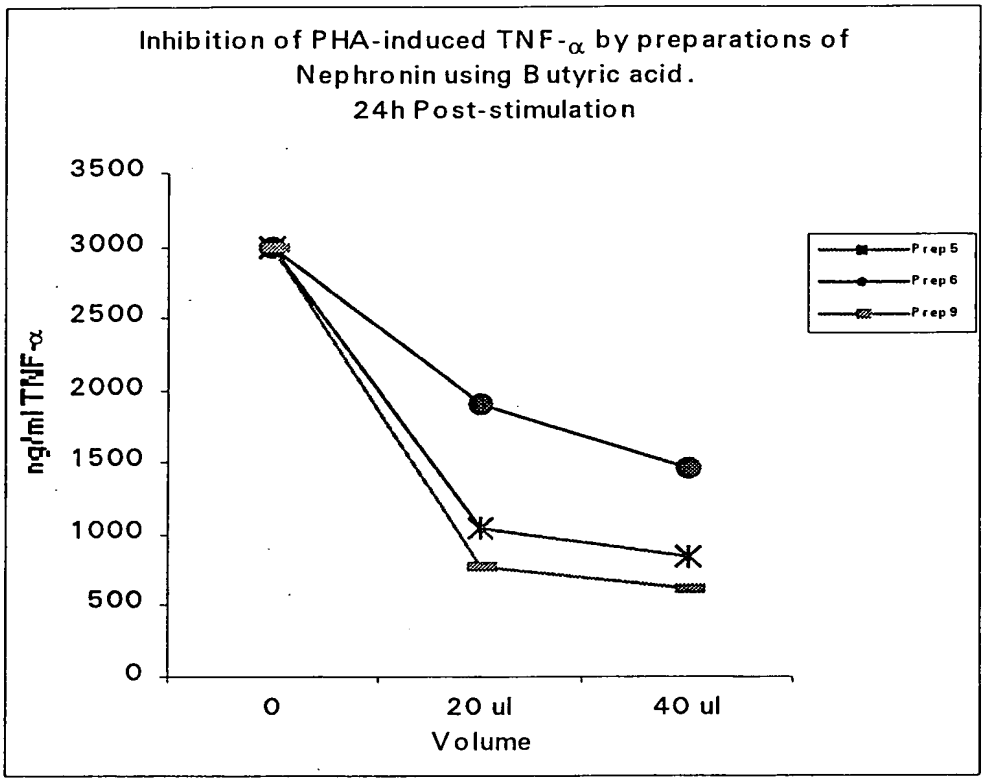
Experiment 17R

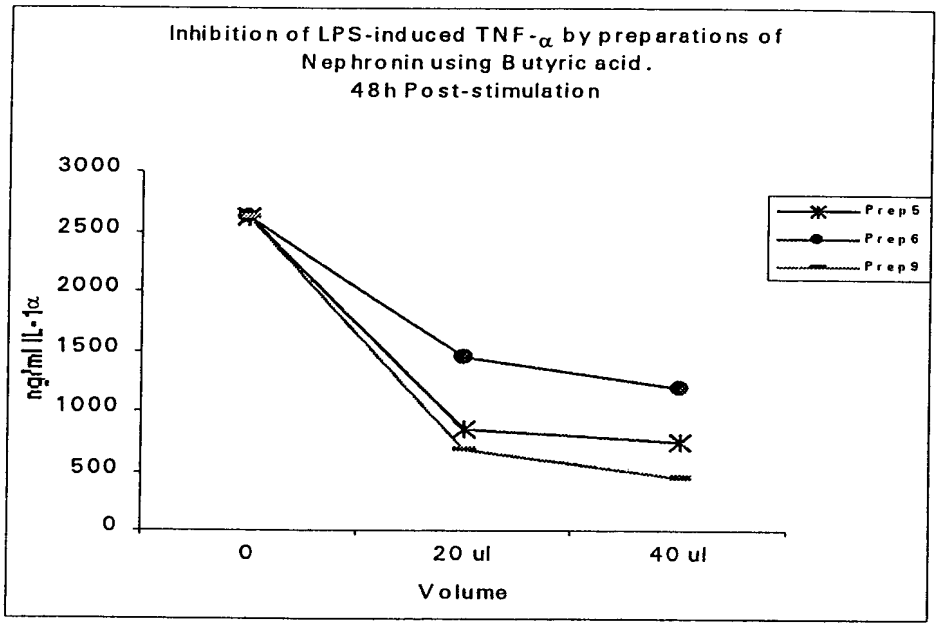
Fig 21



Dose dependent inhibition of PHA-induced TNF-α production by three preparations of Nephronin using Butyric acid in the synthesis procedure.
Prep 5 refers to preparation of Nephronin, by pre-treating Na3Citrate with Butyric acid and the mixture used in the synthesis.
Prep 6 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with butyric acid, the acid soluble material was used in the synthesis procedure.
Prep 9 refers to preparation of Nephronin, where after pre-treatment of Na3Citrate with Butyric acid and removal of the acid soluble material, the remaining solid was used in the synthesis procedure.
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added and then 1ml of PBMC at $1.5x10^6$ cells/ml was added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 17R

Fig 22



Dose dependent inhibition of PHA-induced TNF-α production by three preparations of Nephronin using Butyric acid in the synthesis procedure.
Prep 5 refers to preparation of Nephronin, by pre-treating Na3Citrate with Butyric acid and the mixture used in the synthesis.
Prep 6 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with butyric acid, the acid soluble material was used in the synthesis procedure.
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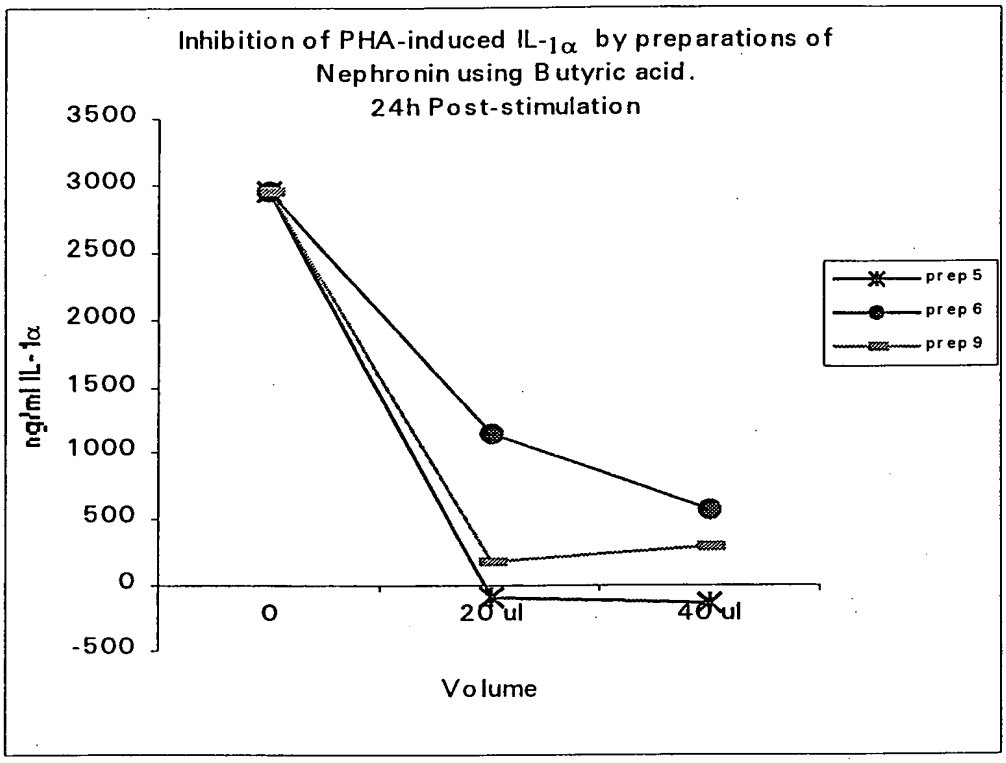
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added and then 1ml of PBMC at $1.5x10^6$ cells/ml was added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 17R

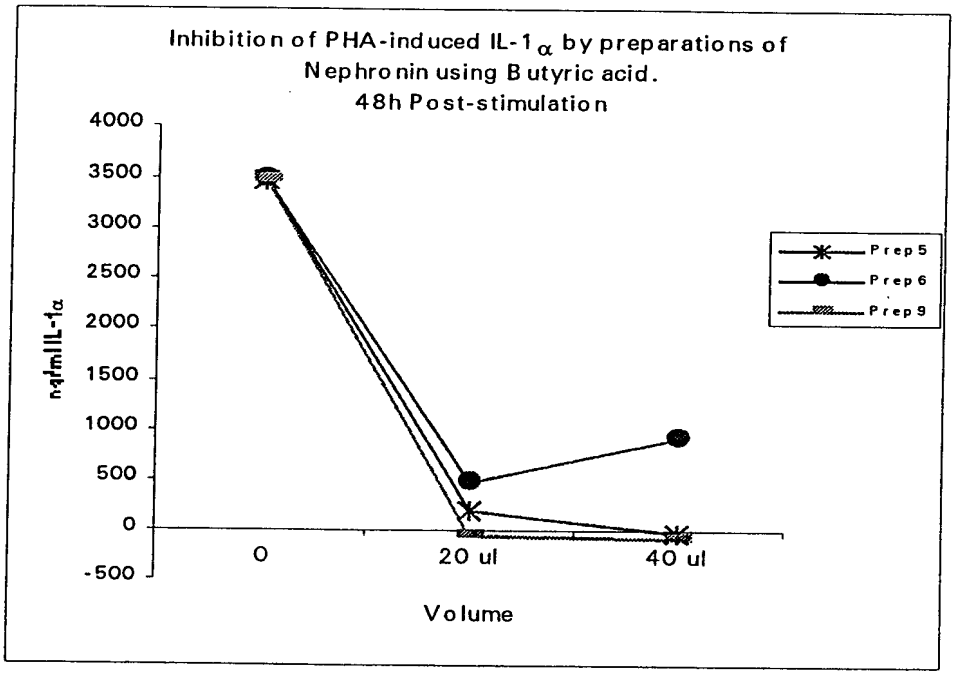
Fig 23



Dose dependent inhibition of PHA-induced IL-1α production by three preparations of Nephronin using Butyric acid in the synthesis procedure.
Prep 5 refers to preparation of Nephronin, by pre-treating Na3Citrate with Butyric acid and the mixture used in the synthesis.
Prep 6 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with butyric acid, the acid soluble material was used in the synthesis procedure.
Prep 9 refers to preparation of Nephronin, where after pre-treatment of Na3Citrate with Butyric acid and removal of the acid soluble material, the remaining solid was used in the synthesis procedure.
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added and then 1ml of PBMC at $1.5x10^6$ cells/ml was added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 17R

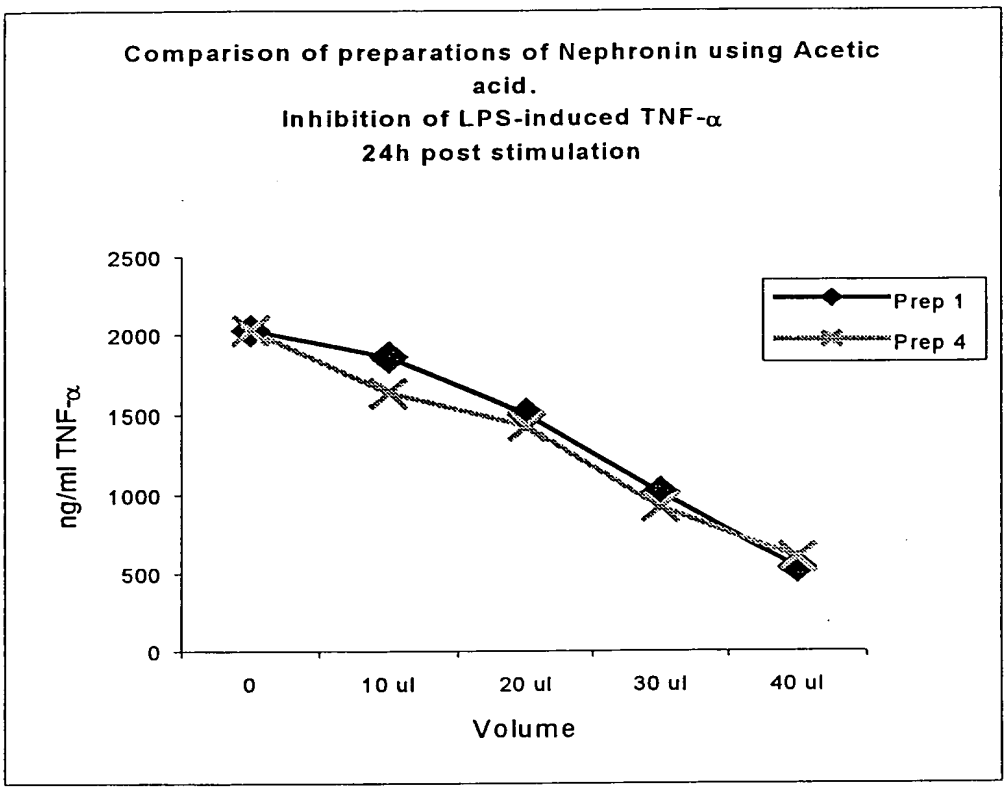
Fig 24



Dose dependent inhibition of PHA-induced IL-1α production by three preparations of Nephronin using Butyric acid in the synthesis procedure.
Prep 5 refers to preparation of Nephronin, by pre-treating Na3Citrate with Butyric acid and the mixture used in the synthesis.
Prep 6 refers to preparation of Nephronin, where after pre-treatment of Na3citrate with butyric acid, the acid soluble material was used in the synthesis procedure.
Prep 9 refers to preparation of Nephronin, where after pre-treatment of Na3Citrate with Butyric acid and removal of the acid soluble material, the remaining solid was used in the synthesis procedure.
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added and then 1ml of PBMC at 1.5×10^6 cells/ml was added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 18

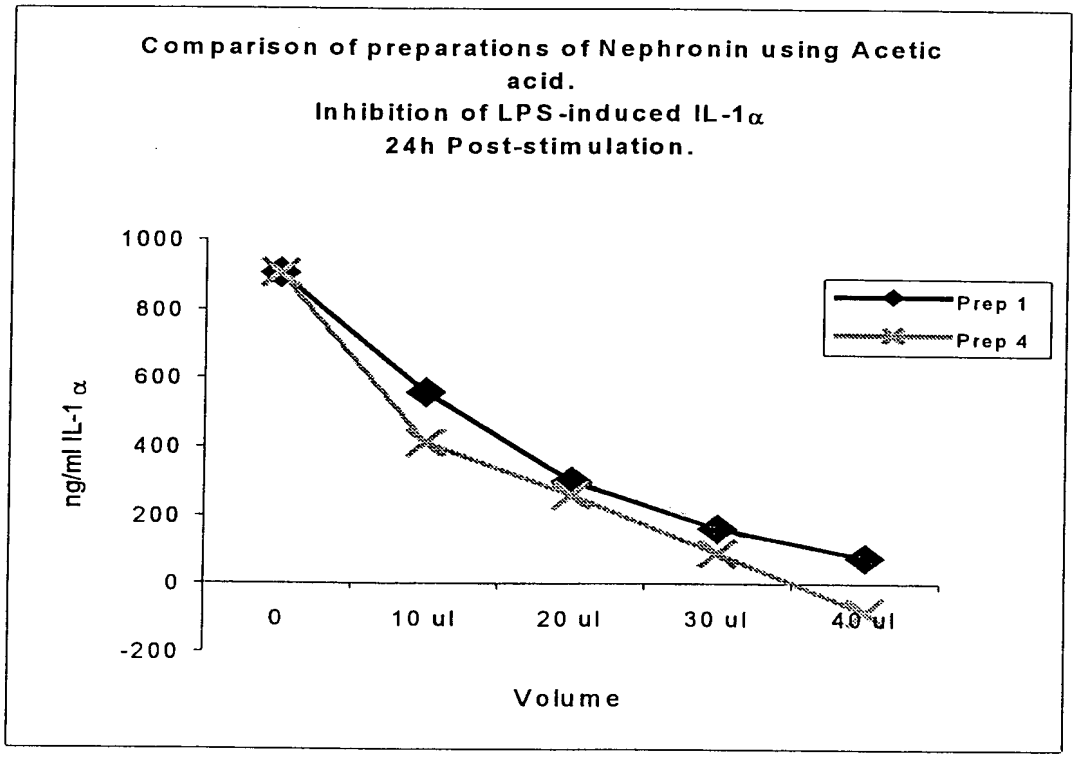
Fig 1



Dose dependent inhibition of LPS-induced TNF-α production by two preparations of Nephronin using Acetic acid in the synthesis procedure.
Preparations 1 and 4 are identical except that the solvent layer in preparation 1 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 18

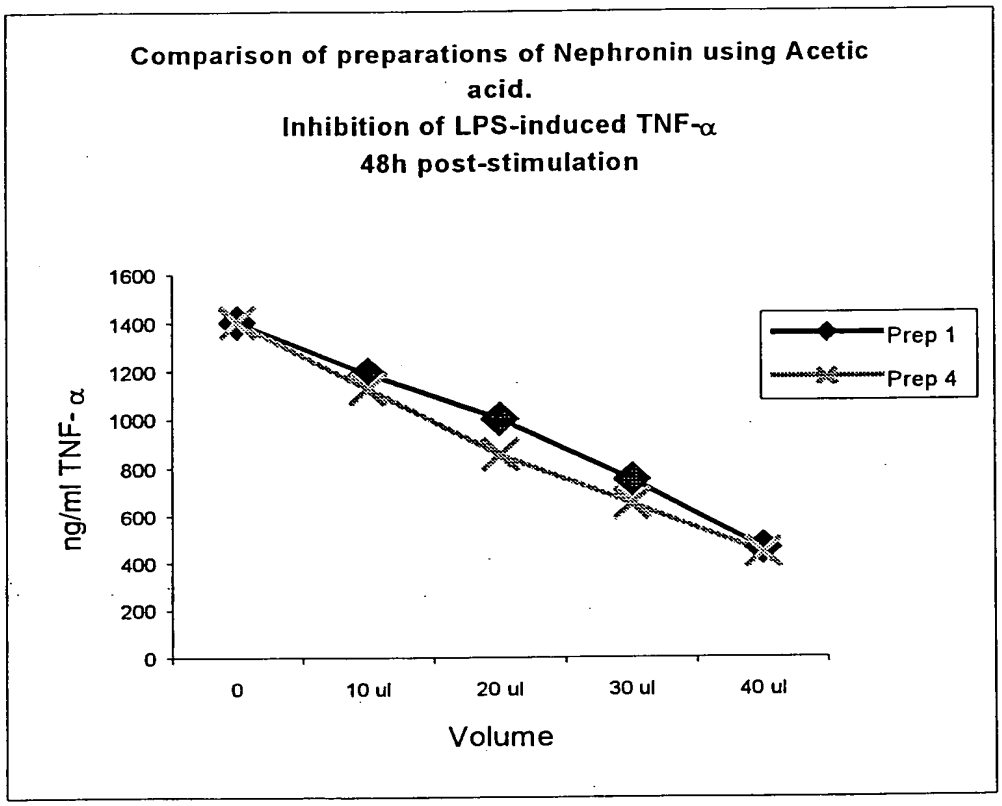
Fig 2



Dose dependent inhibition of LPS-induced IL-1α production by two preparations of Nephronin using Acetic acid in the synthesis procedure.
Preparations 1 and 4 are identical except that the solvent layer in preparation 1 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 18

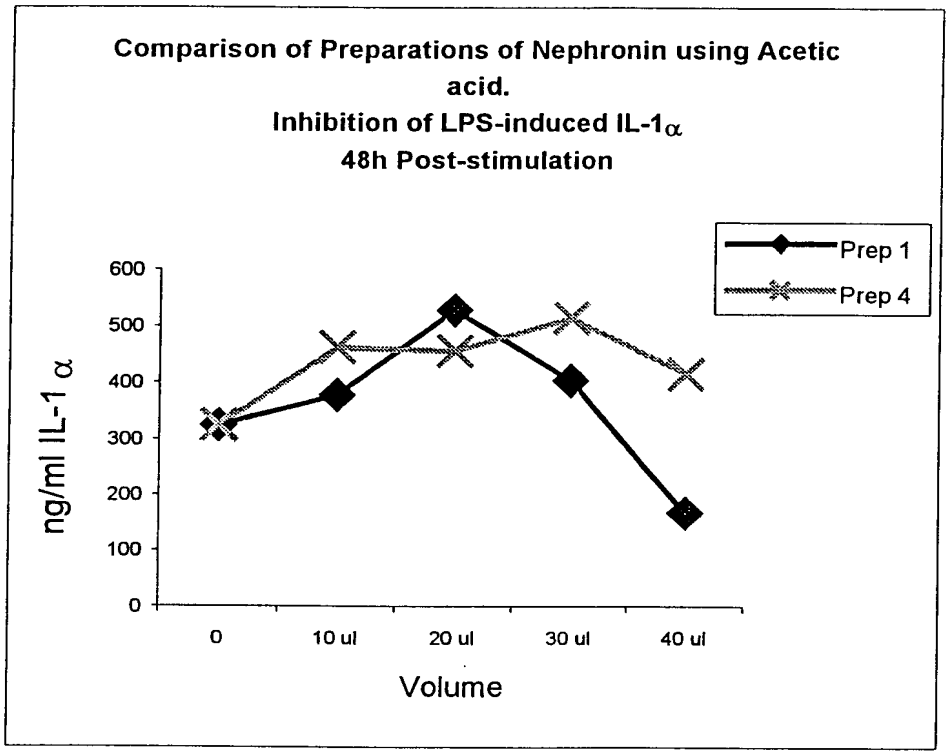
Fig 3



Dose dependent inhibition of LPS-induced TNF-α production by two preparations of Nephronin using Acetic acid in the synthesis procedure.
Preparations 1 and 4 are identical except that the solvent layer in preparation 1 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 18

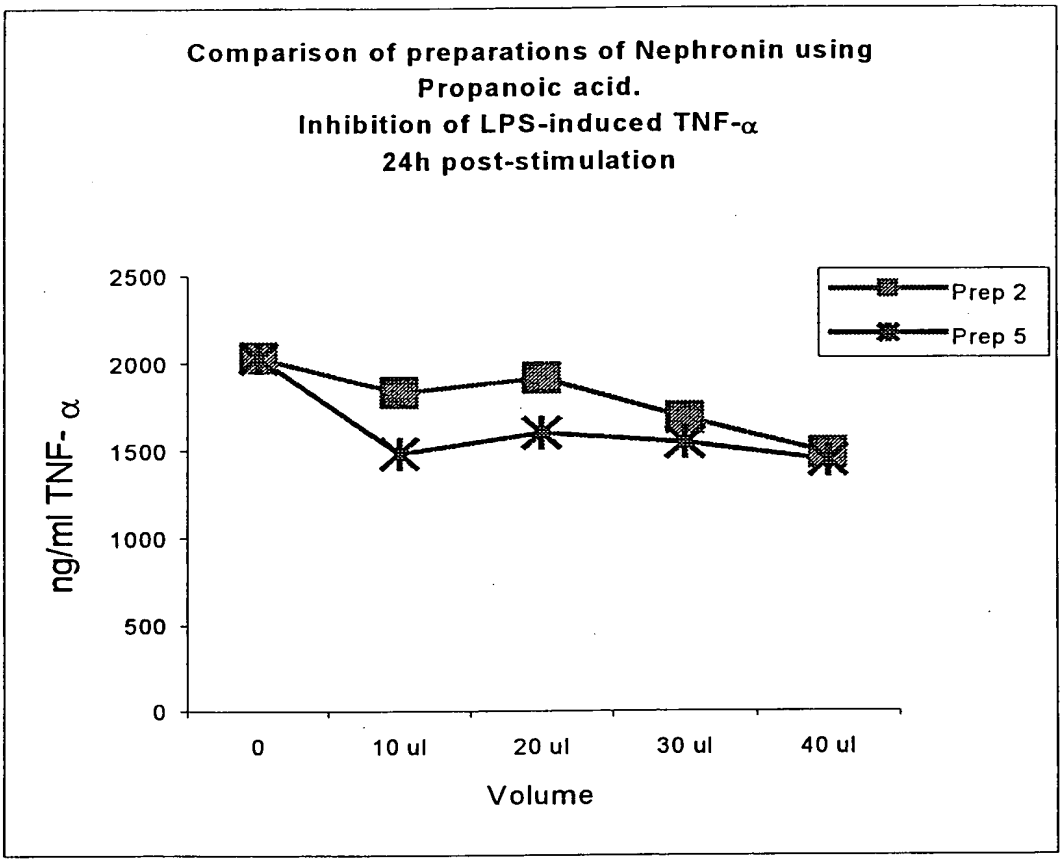
Fig 4



Dose dependent inhibition of LPS-induced IL-1α production by two preparations of Nephronin using Acetic acid in the synthesis procedure.
Preparations 1 and 4 are identical except that the solvent layer in preparation 1 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plate. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 18

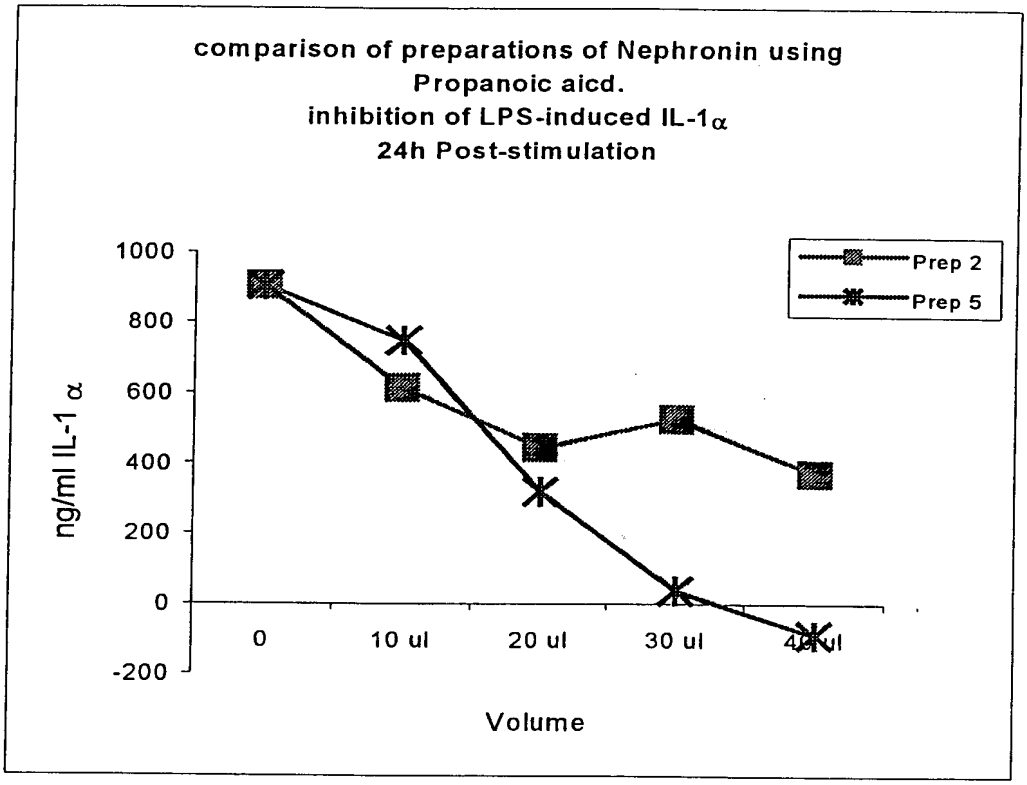
Fig 5



Dose dependent inhibition of LPS-induced TNF-α production by two preparations of Nephronin using Propanoic acid in the synthesis procedure.
Preparations 2 and 5 are identical except that the solvent layer in preparation 2 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 18

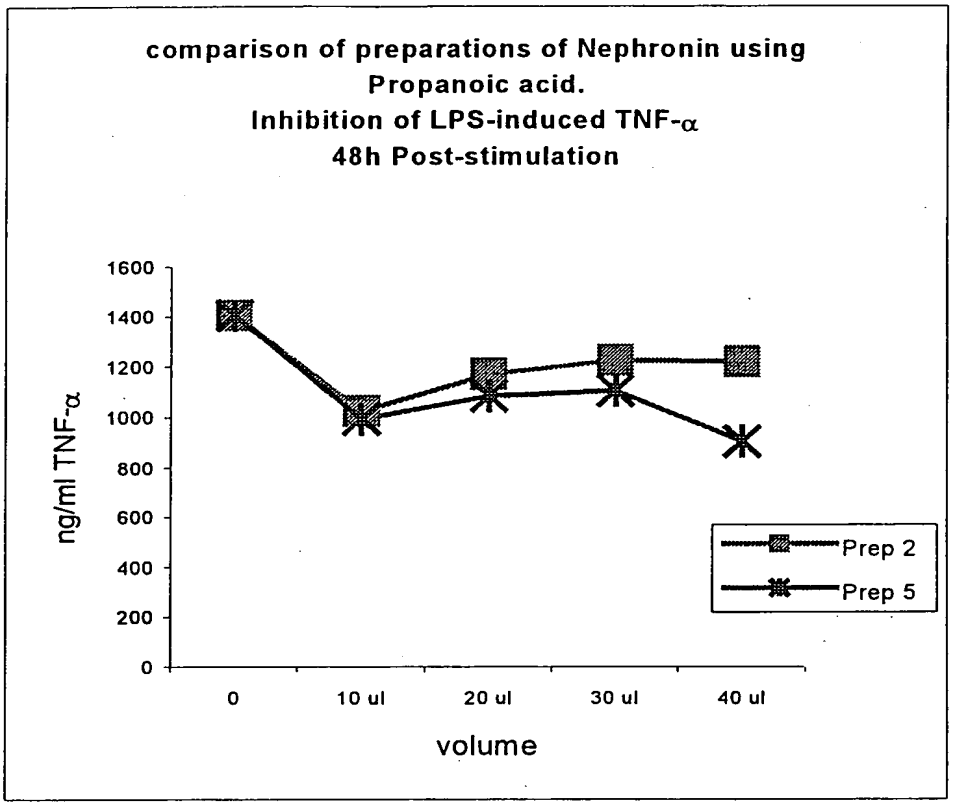
Fig 6



Dose dependent inhibition of LPS-induced IL-1α production by two preparations of Nephronin using Propanoic acid in the synthesis procedure.
Preparations 2 and 5 are identical except that the solvent layer in preparation 2 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 18

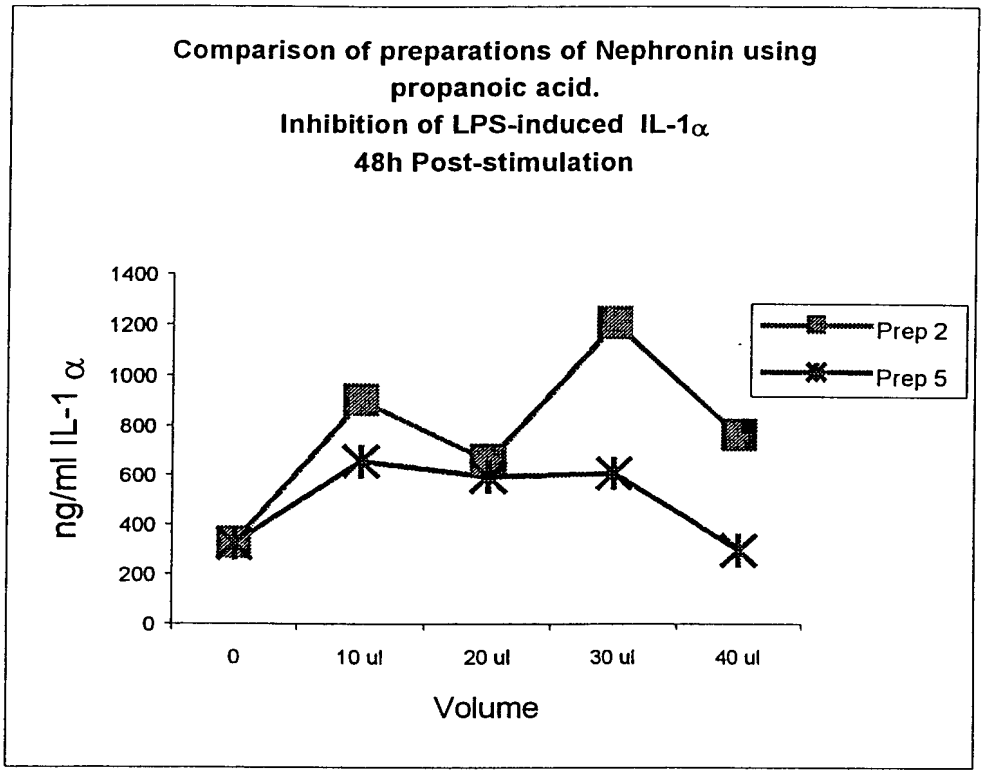
Fig 7



Dose dependent inhibition of LPS-induced TNF-α production by two preparations of Nephronin using Propanoic acid in the synthesis procedure.
Preparations 2 and 5 are identical except that the solvent layer in preparation 2 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 18

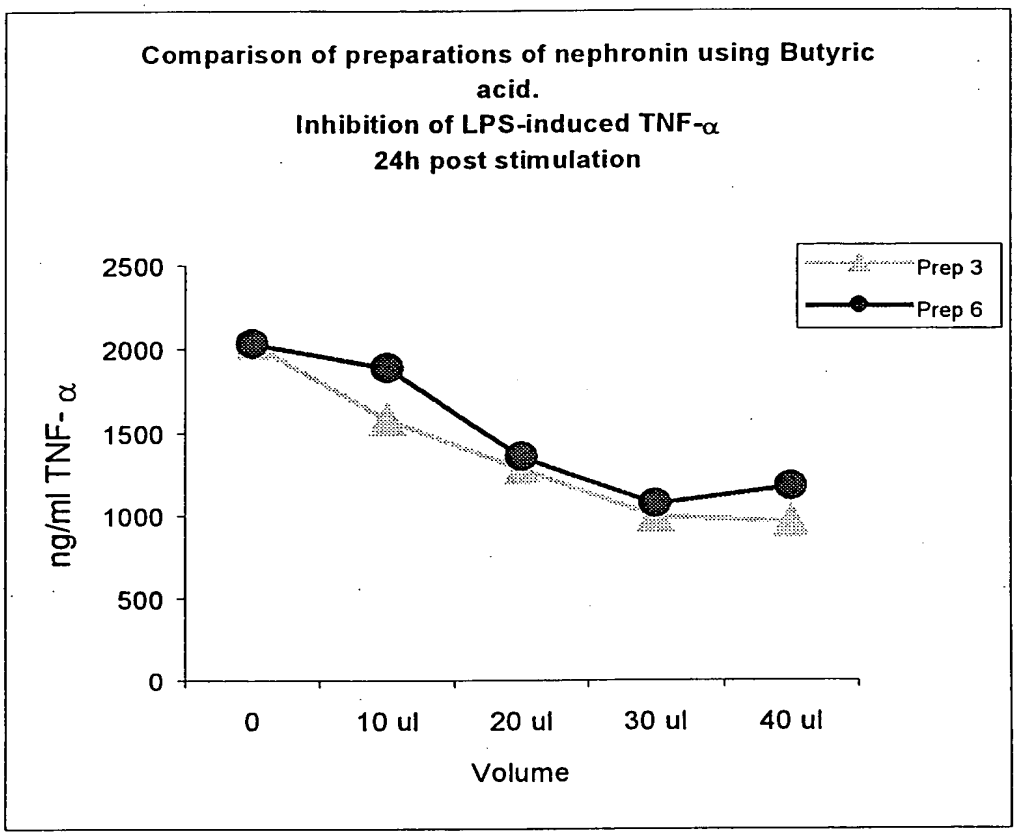
Fig 8



Dose dependent inhibition of LPS-induced IL-1α production by two preparations of Nephronin using Propanoic acid in the synthesis procedure.
Preparations 2 and 5 are identical except that the solvent layer in preparation 2 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 18

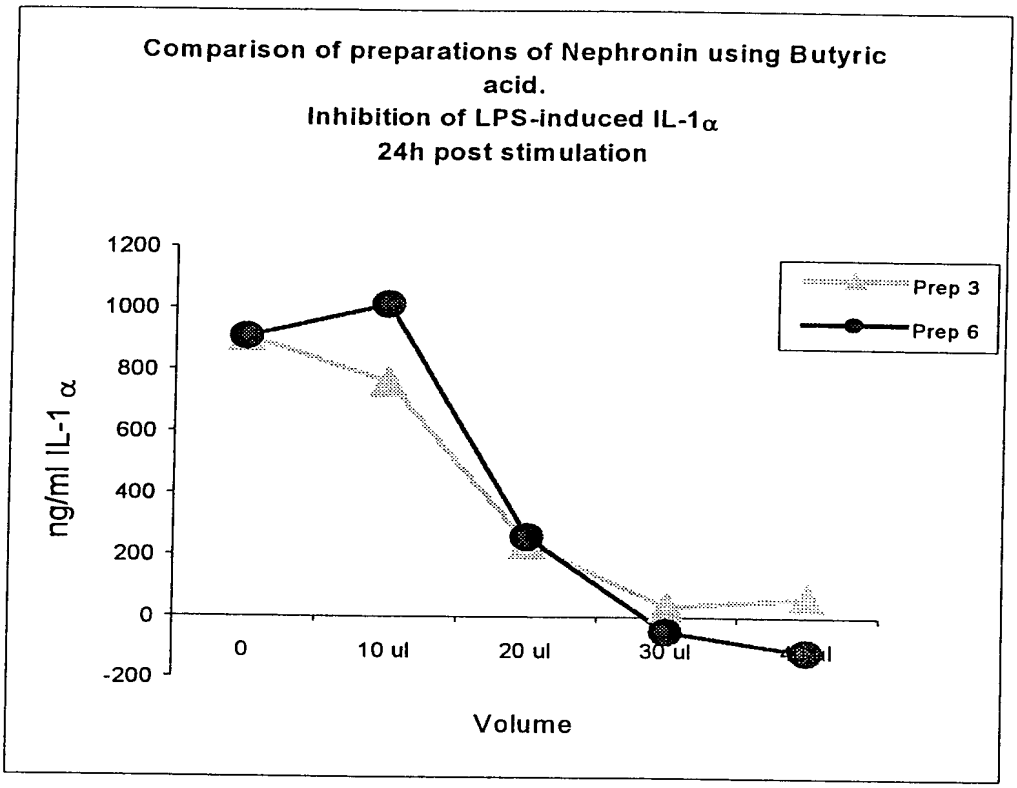
Fig 9



Dose dependent inhibition of LPS-induced TNF-α production by two preparations of Nephronin using Butyric acid in the synthesis procedure.
Preparations 3 and 6 are identical except that the solvent layer in preparation 3 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 18

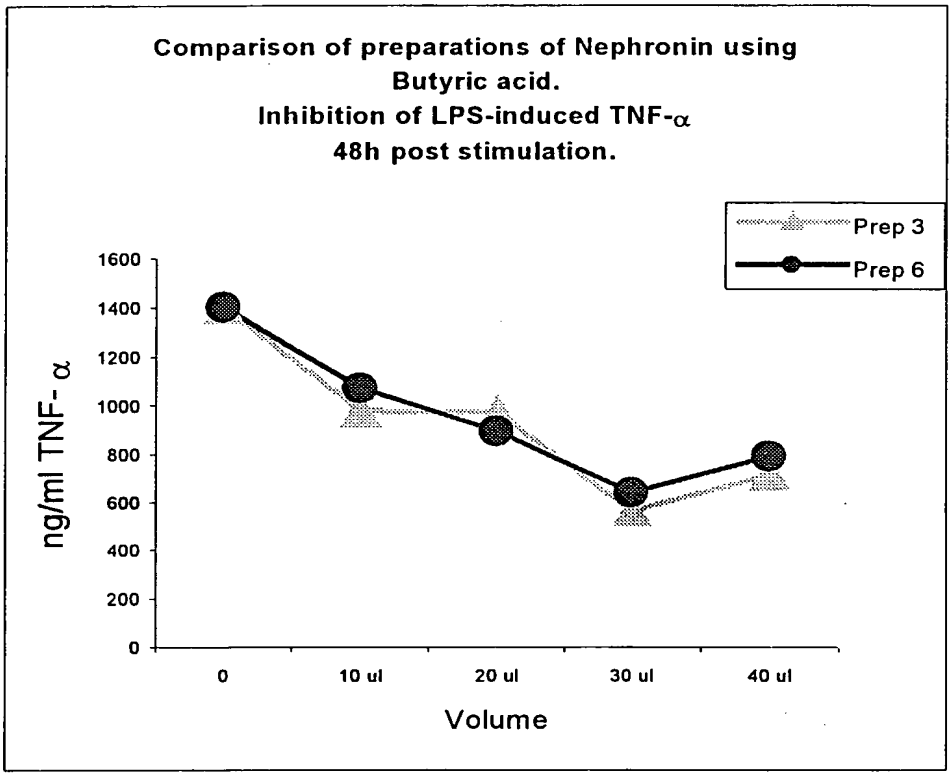
Fig 10



Dose dependent inhibition of LPS-induced IL-1α production by two preparations of Nephronin using Butyric acid in the synthesis procedure.
Preparations 3 and 6 are identical except that the solvent layer in preparation 3 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 18

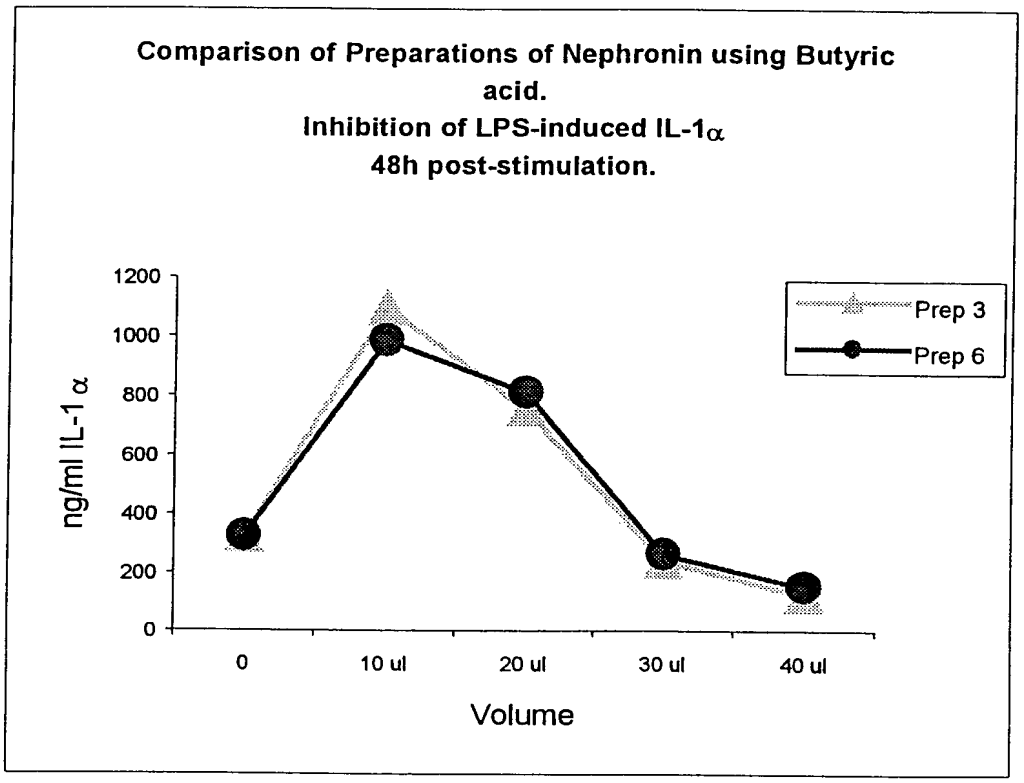
Fig 11



Dose dependent inhibition of LPS-induced TNF-α production by two preparations of Nephronin using Butyric acid in the synthesis procedure.
Preparations 3 and 6 are identical except that the solvent layer in preparation 3 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 18

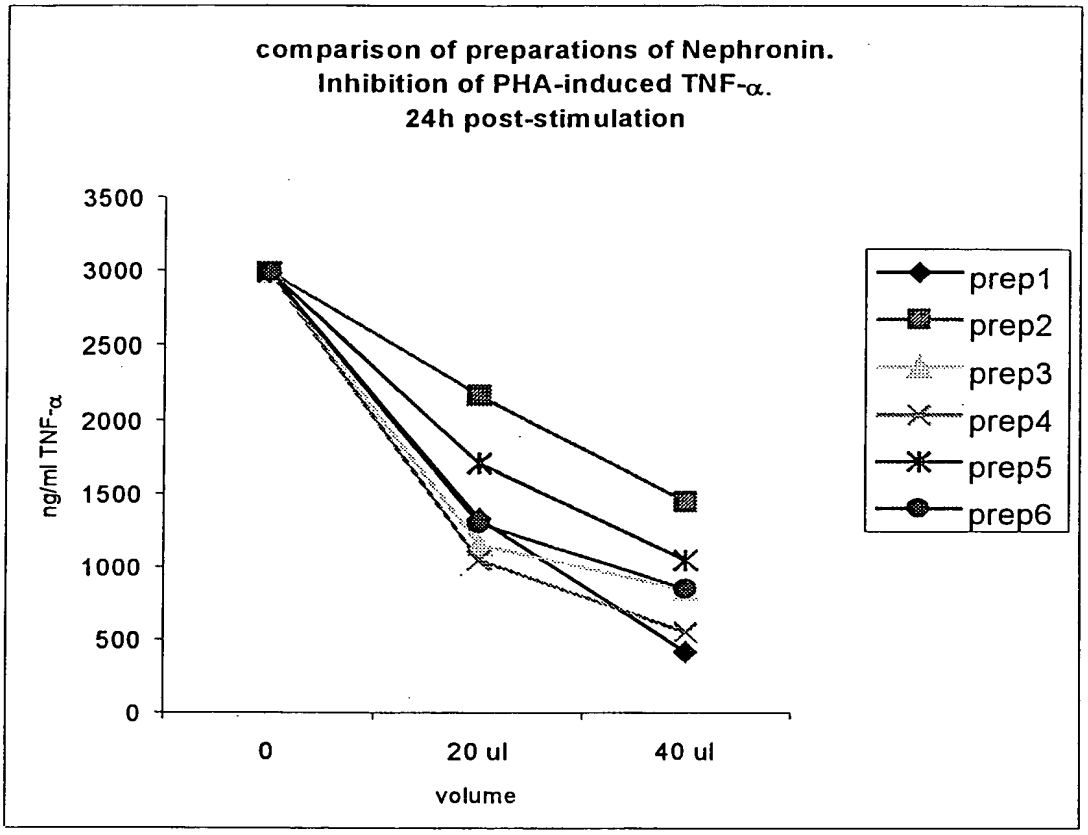
Fig 12



Dose dependent inhibition of LPS-induced IL-1α production by two preparations of Nephronin using Butyric acid in the synthesis procedure.
Preparations 3 and 6 are identical except that the solvent layer in preparation 3 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting LPS-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml LPS was then added to each well. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 18

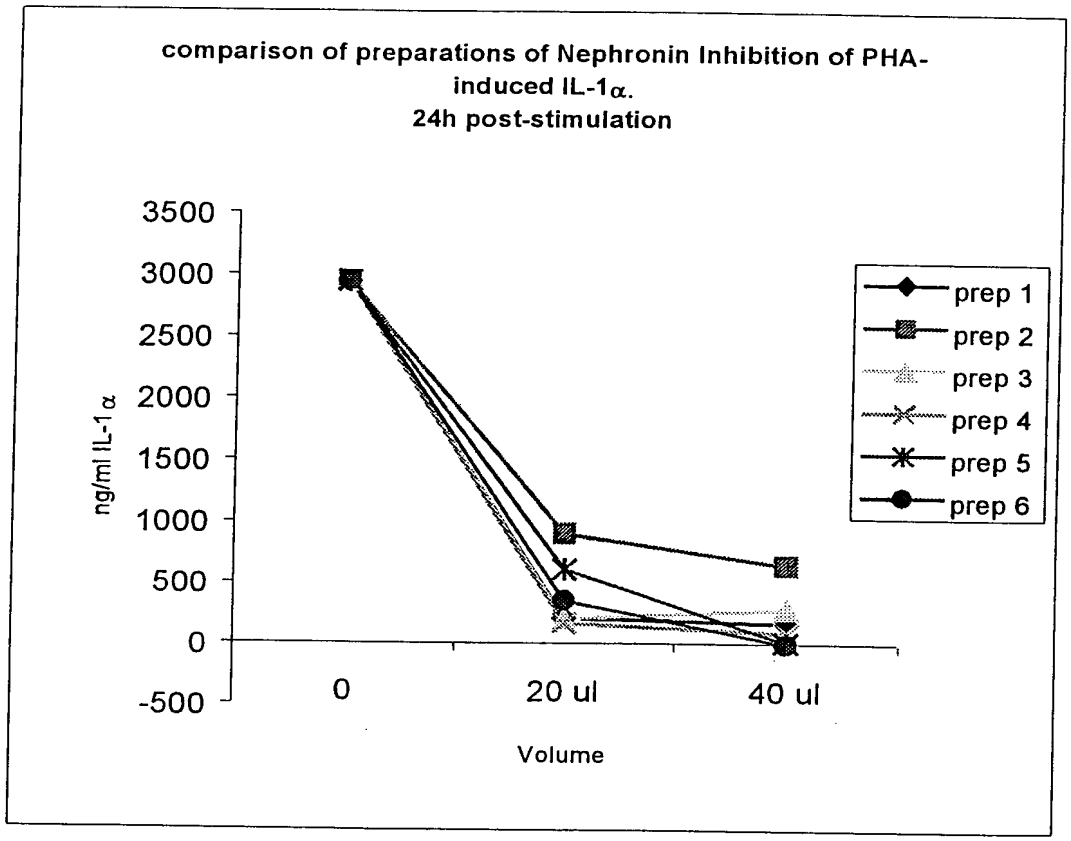
Fig 13



Dose dependent inhibition of PHA-induced TNF-α production by six preparations of Nephronin.
All preparations were synthesised identically except that the solvent layer in preparations 1, 2 and 3 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added followed by 1ml of PBMC at $1.5x10^6$ cells/ml. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 18

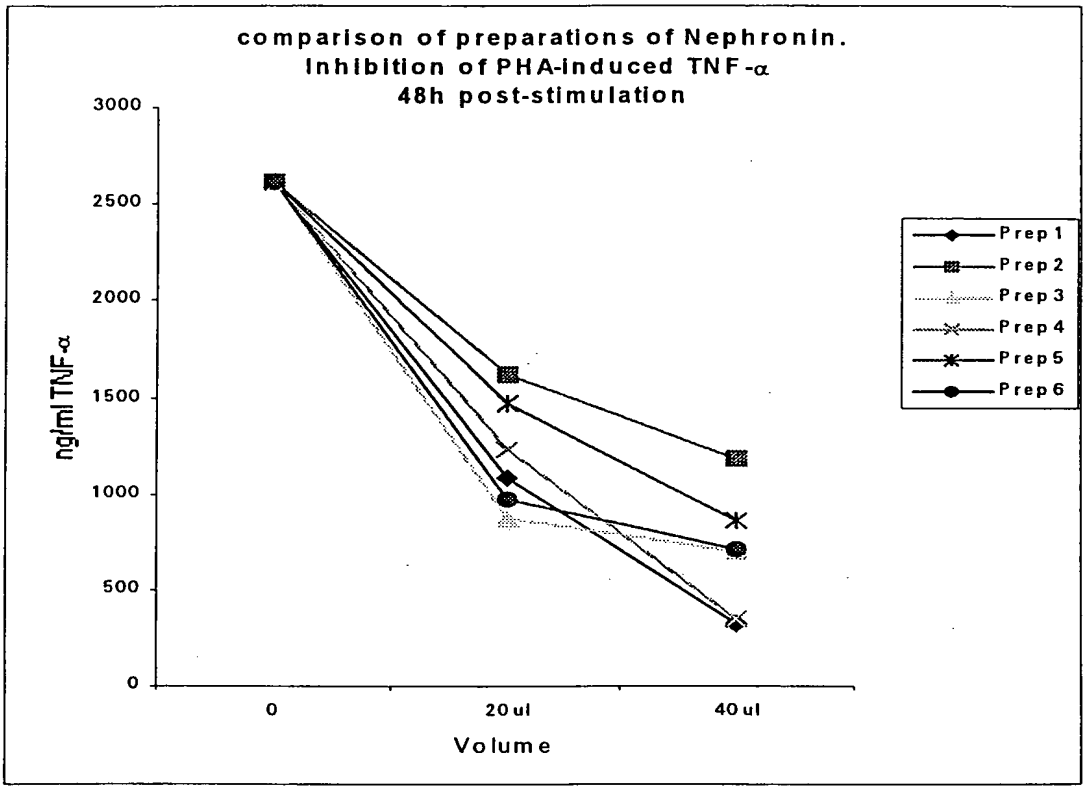
Fig 14



Dose dependent inhibition of PHA-induced IL-1α production by six preparations of Nephronin.
All preparations were synthesised identically except that the solvent layer in preparations 1, 2 and 3 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added followed by 1ml of PBMC at 1.5×10^6 cells/ml. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 18

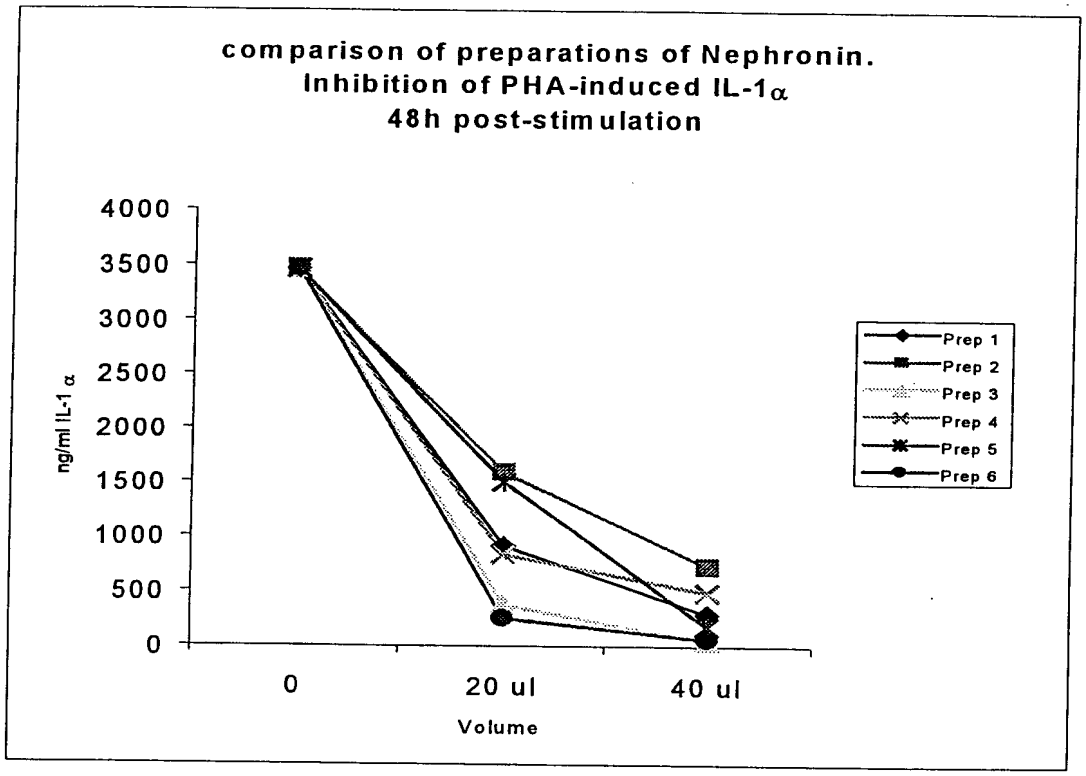
Fig 15



Dose dependent inhibition of PHA-induced TNF-α production by six preparations of Nephronin.
All preparations were synthesised identically except that the solvent layer in preparations 1, 2 and 3 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced TNF-α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added followed by 1ml of PBMC at 1.5×10^6 cells/ml. The cells were harvested at the indicated time and the concentration of TNF-α measured by sandwich ELISA.

Experiment 18

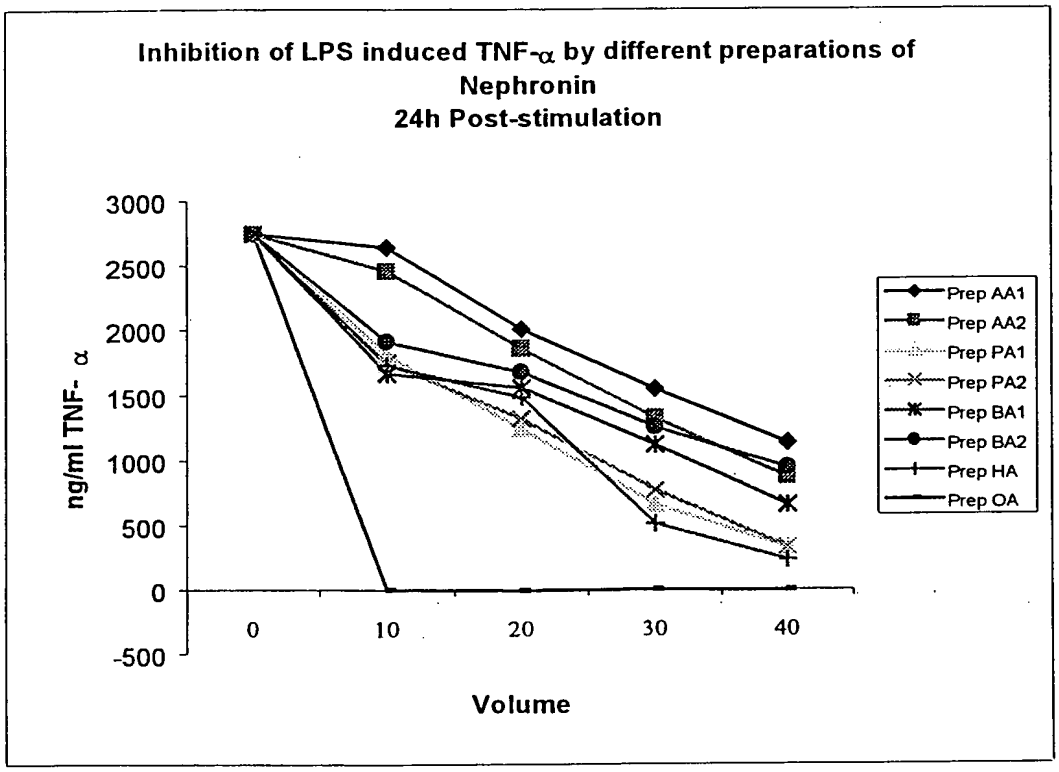
Fig 16



Dose dependent inhibition of PHA-induced IL-1α production by six preparations of Nephronin.
All preparations were synthesised identically except that the solvent layer in preparations 1, 2 and 3 was removed.
An increasing volume of each preparation was used to test their potency in inhibiting PHA-induced IL-1α production.
The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. To each well 5μg of PHA was added followed by 1ml of PBMC at 1.5×10^6 cells/ml. The cells were harvested at the indicated time and the concentration of IL-1α measured by sandwich ELISA.

Experiment 21

Fig 1

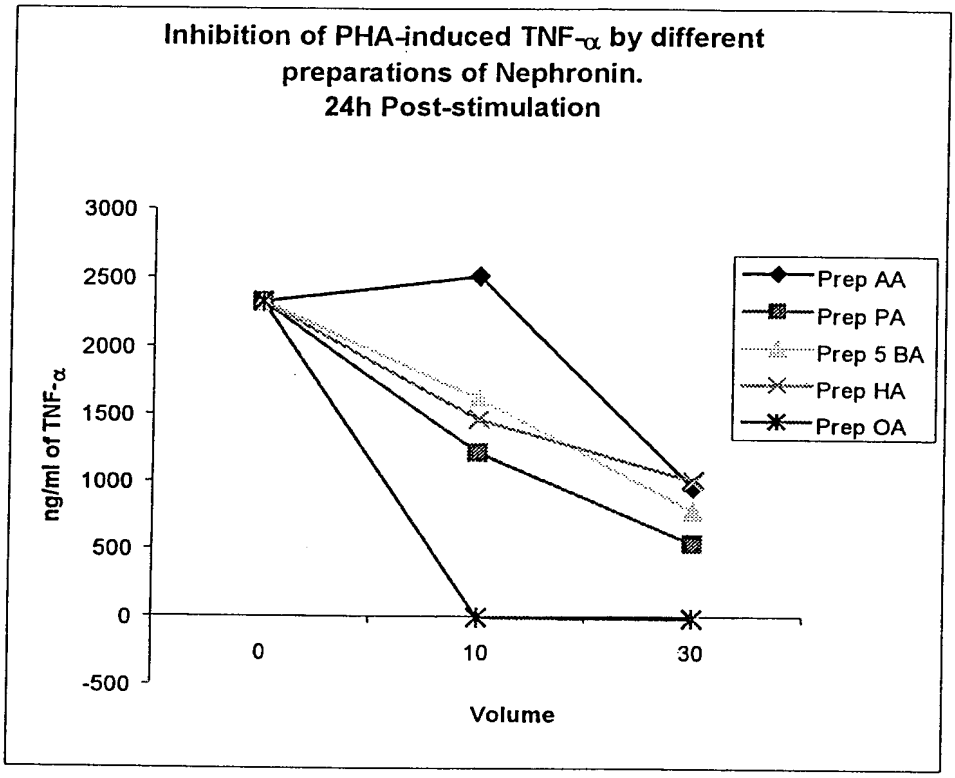


Comparison of dose dependent inhibition profile LPS-induced TNF-α production by eight preparations of Nephronin. In the cases of AA, BA and PA, duplicate preparations were prepared to test for differences in response in a given biological system.
The lettering beside each preparation indicates the acid used in the synthesis procedure.

In each case, the indicated volume of the each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 21

Fig 2

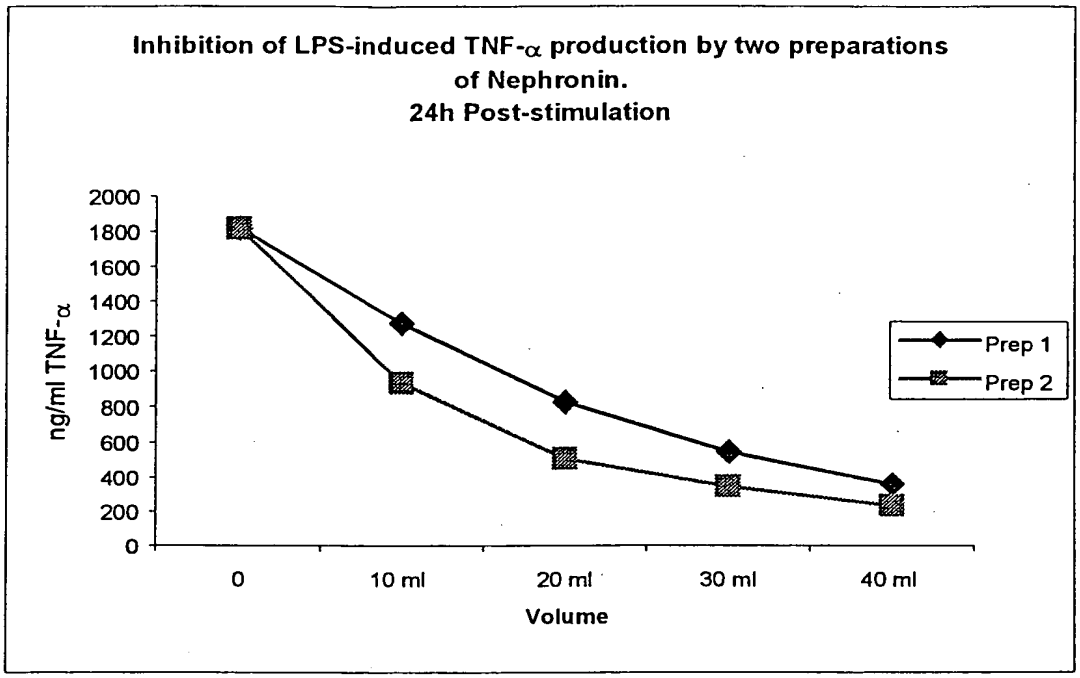


A dose dependent inhibition profile PHA-induced TNF-α production by five preparations of Nephronin.
The lettering beside each preparation indicates the acid used in the synthesis procedure.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 5μg of PHA was added to all wells followed by the addition of 1 ml of PBMC at $1.5x10^6$ cells/ml per well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 22

Fig 1



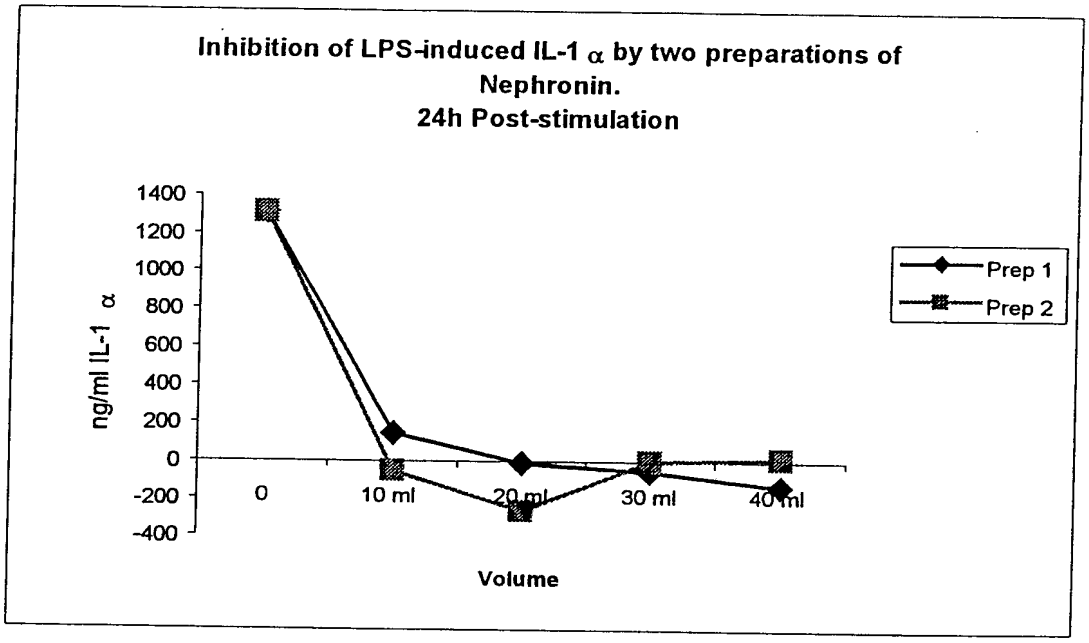
Dose dependent inhibition profile of LPS-induced TNF-α production by two different preparations of Nephronin.

In both cases, Na3Citrate was first treated with excess acetic acid (prep1) or propanoic acid (prep 2). The resulting white solid material was then used in the synthesis procedure.

The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml per well containing 1ng/ml LPS was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 22

Fig 2



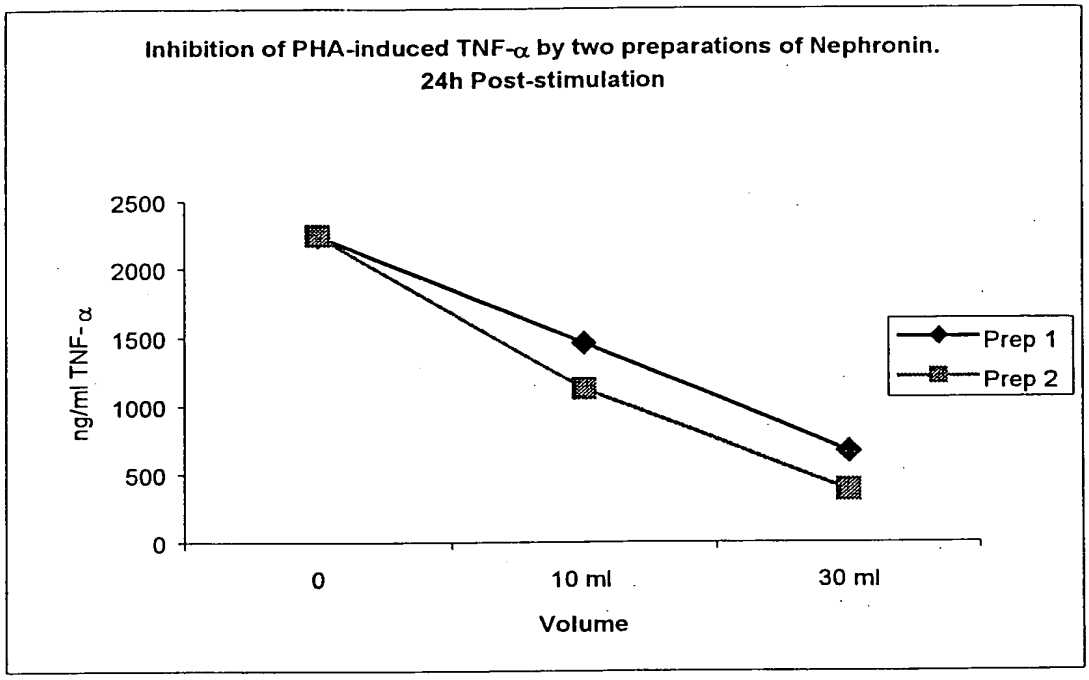
Dose dependent inhibition profile of LPS-induced IL-1α production by two different preparations of Nephronin.

In both cases, Na3Citrate was first treated with excess acetic acid (prep1) or propanoic acid (prep 2). The resulting white solid material was then used in the synthesis procedure.

The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml per well containing 1ng/ml LPS was then added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA.

Experiment 22

Fig 3



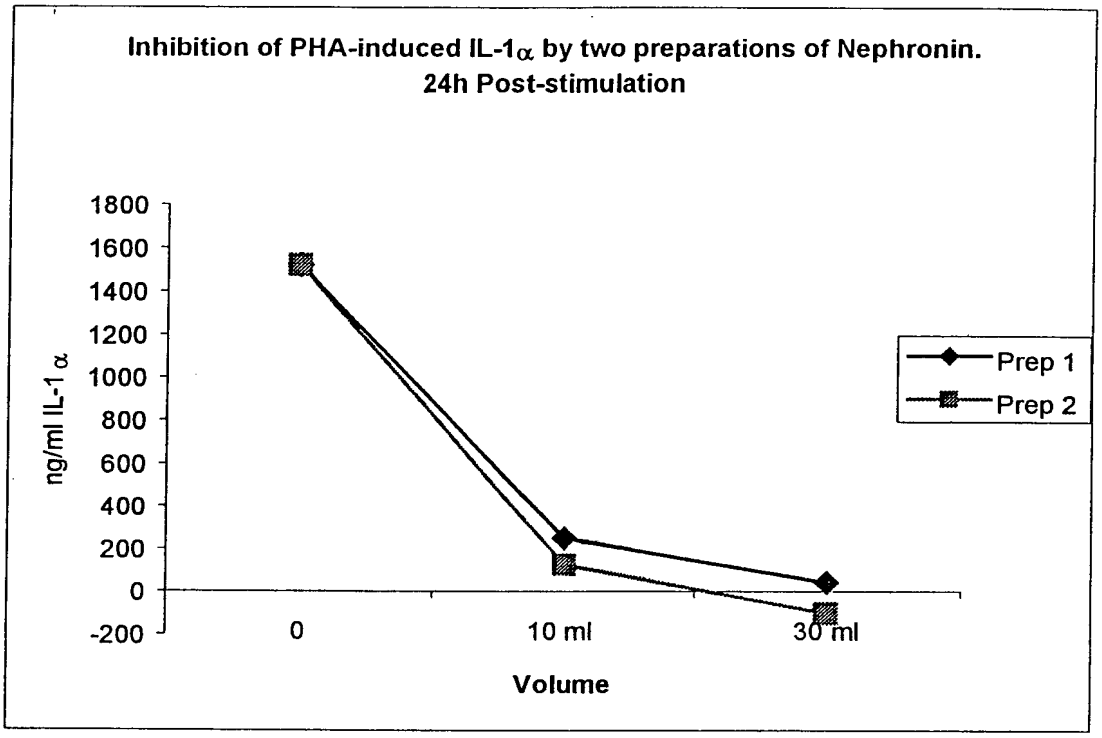
Dose dependent inhibition profile of PHA-induced TNF-α production by two different preparations of Nephronin.

In both cases, Na3Citrate was first treated with excess acetic acid (prep1) or propanoic acid (prep 2). The resulting white solid material was then used in the synthesis procedure.

The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 5μg of PHA was added to each well followed by the addition of 1 ml of PBMC at 1.5×10^6 cells/ml. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 22

Fig 4



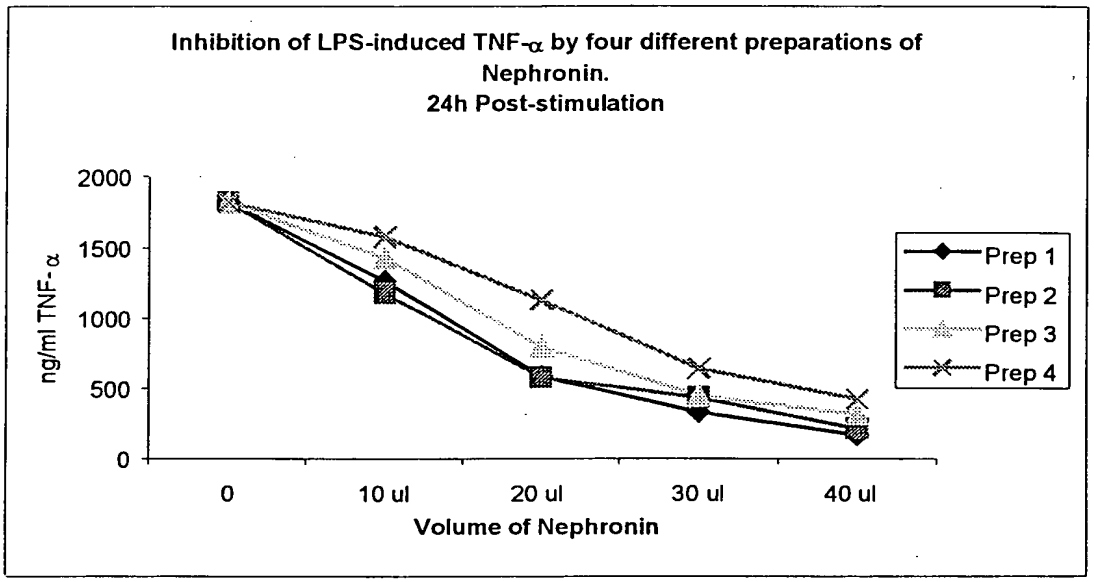
Dose dependent inhibition profile of PHA-induced IL-1α production by two different preparations of Nephronin.

In both cases, Na3Citrate was first treated with excess acetic acid (prep1) or propanoic acid (prep 2). The resulting white solid material was then used in the synthesis procedure.

The indicated volume of each preparation was added to chambers of 24 well tissue culture plates. 5μg of PHA was added to each well followed by the addition of 1 ml of PBMC at $1.5x10^6$ cells/ml. The cells were harvested 24h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA.

Experiment 23

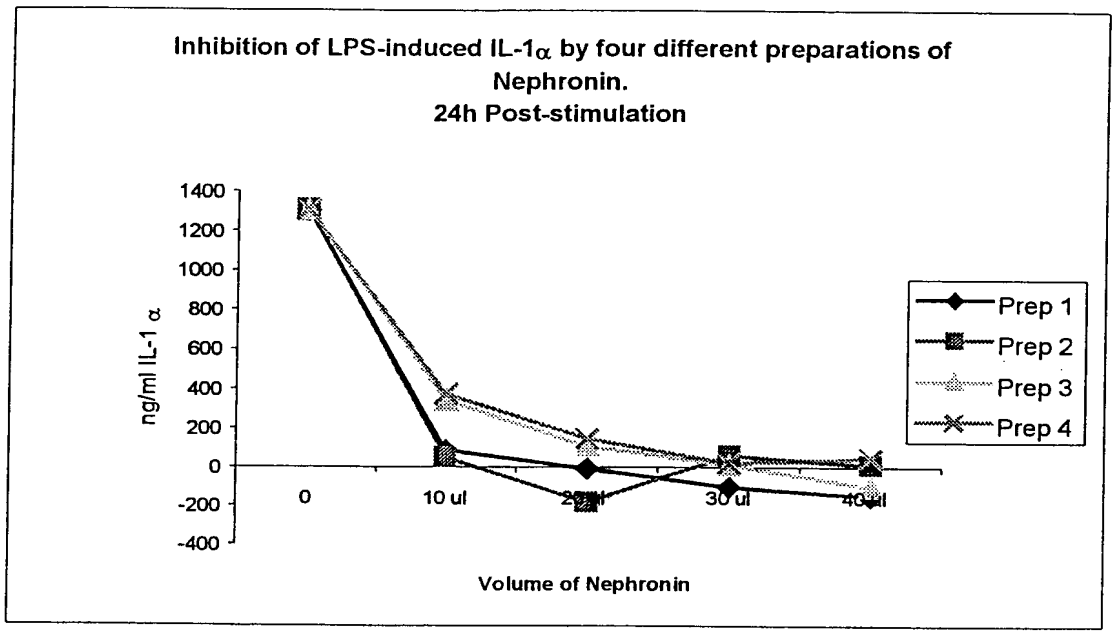
Fig 1



Dose dependent inhibition profile of LPS-induced TNF-α production by four different preparations of Nephronin.
In each case Na3Citrate was first treated with excess acetic acid. The resulting white solid material, following centrifugation and aspiration of the liquid was used in the synthesis procedure where different acids were utilised in the synthesis procedure.
Preparation 1 refers to synthesis procedure where propanoic acid was used. Preparations 2, 3 and 4 refer to synthesis procedures where Butyric acid, Hexanoic acid and Octanoic acids were used respectively.
The indicated volume from each preparation was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml per well containing 1ng/ml LPS was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA

Experiment 23

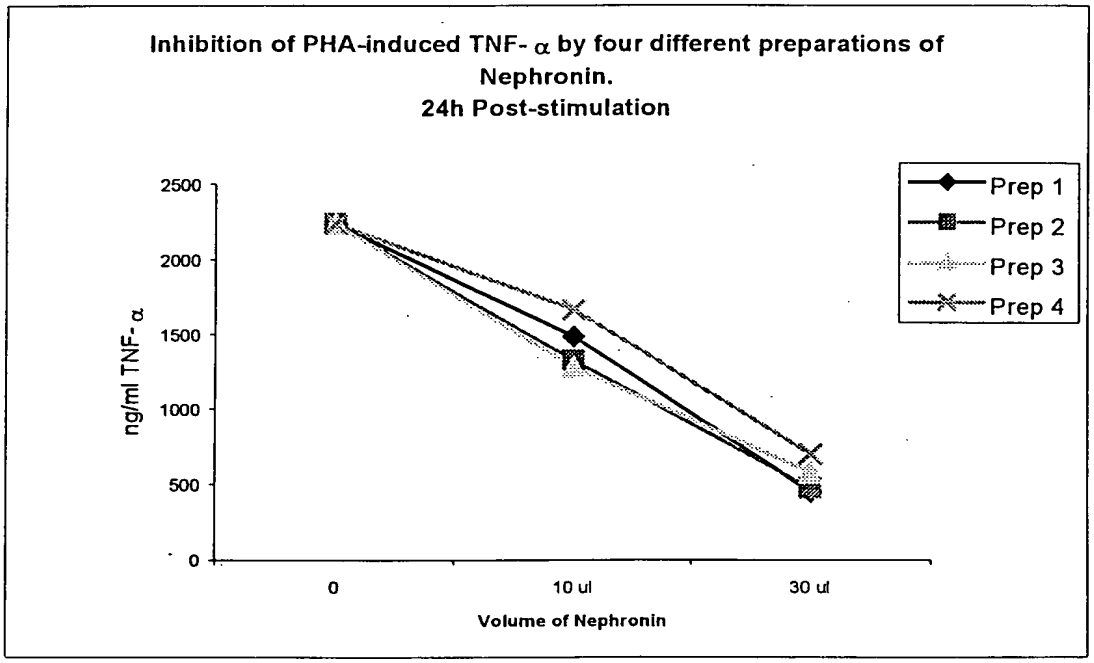
Fig 2



Dose dependent inhibition profile of LPS-induced IL-1α production by four different preparations of Nephronin.
In each case Na3Citrate was first treated with excess acetic acid. The resulting white solid material, following centrifugation and aspiration of the liquid was used in the synthesis procedure where different acids were utilised in the synthesis procedure.
Preparation 1 refers to synthesis procedure where propanoic acid was used. Preparations 2, 3 and 4 refer to synthesis procedures where Butyric acid, Hexanoic acid and Octanoic acids were used respectively.
The indicated volume from each preparation was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml per well containing 1ng/ml LPS was then added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA

Experiment 23

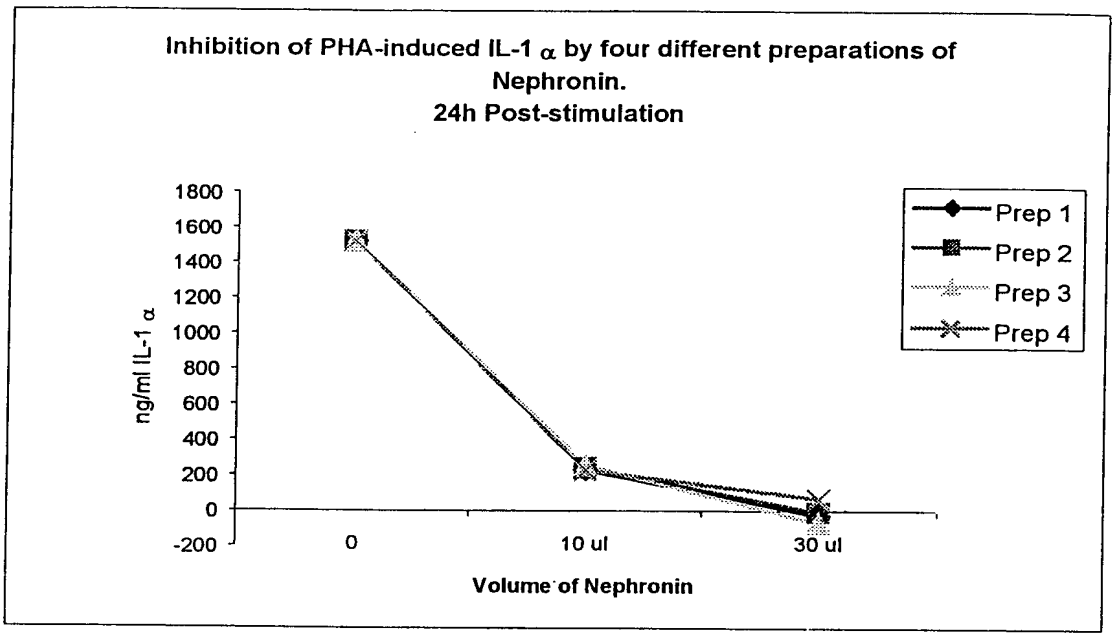
Fig 3



Dose dependent inhibition profile of PHA-induced TNF-α production by four different preparations of Nephronin.
In each case Na3Citrate was first treated with excess acetic acid. The resulting white solid material, following centrifugation and aspiration of the liquid was used in the synthesis procedure where different acids were utilised in the synthesis procedure.
Preparation 1 refers to synthesis procedure where propanoic acid was used. Preparations 2, 3 and 4 refer to synthesis procedures where Butyric acid, Hexanoic acid and Octanoic acids were used respectively.
The indicated volume from each preparation was added to chambers of 24 well tissue culture plates. 5μg of PHA was added to the each well followed by 1 ml of PBMC at $1.5x10^6$ cells/ml. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA

Experiment 23

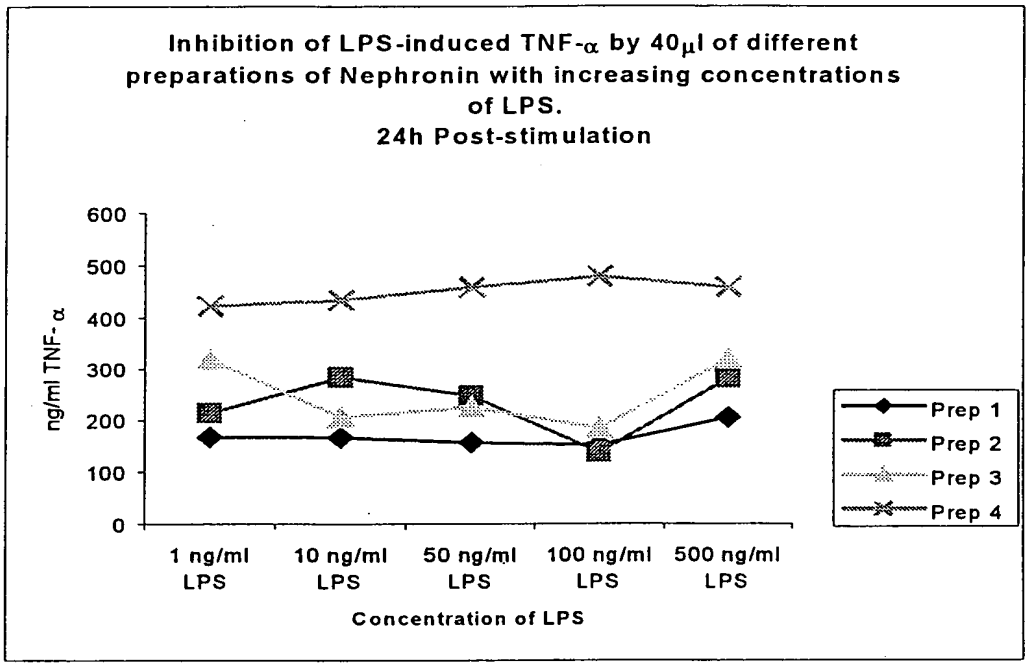
Fig 4



Dose dependent inhibition profile of PHA-induced IL-1α production by four different preparations of Nephronin.
In each case Na3Citrate was first treated with excess acetic acid. The resulting white solid material, following centrifugation and aspiration of the liquid was used in the synthesis procedure where different acids were utilised in the synthesis procedure.
Preparation 1 refers to synthesis procedure where propanoic acid was used. Preparations 2, 3 and 4 refer to synthesis procedures where Butyric acid, Hexanoic acid and Octanoic acids were used respectively.
The indicated volume from each preparation was added to chambers of 24 well tissue culture plates. 5μg of PHA was added to the each well followed by 1 ml of PBMC at $1.5x10^6$ cells/ml. The cells were harvested 24h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA

Experiment 23

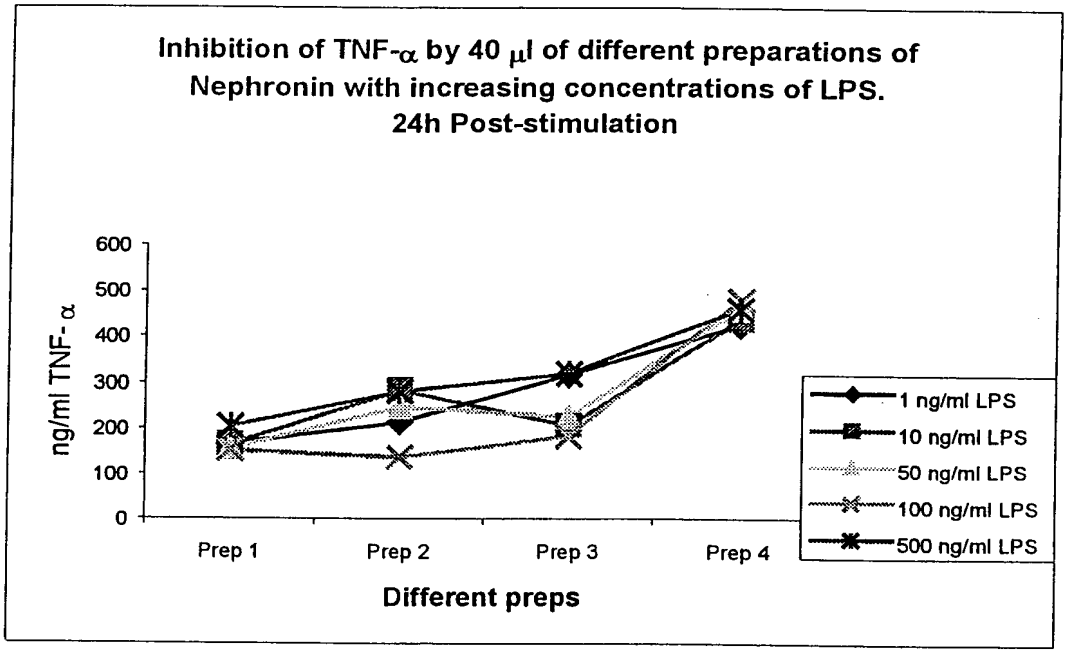
Fig 5



Increasing concentrations of LPS were used to stimulate PBMC. 40μl of each preparation was used in the inhibition of LPS-induced TNF-α production.
In each case 40μl of each preparation was added to chambers of the tissue culture plates containing LPS that would give the indicated concentration in 1ml.
1 ml of PBMC at $1.5x10^6$ cells/ml was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 23

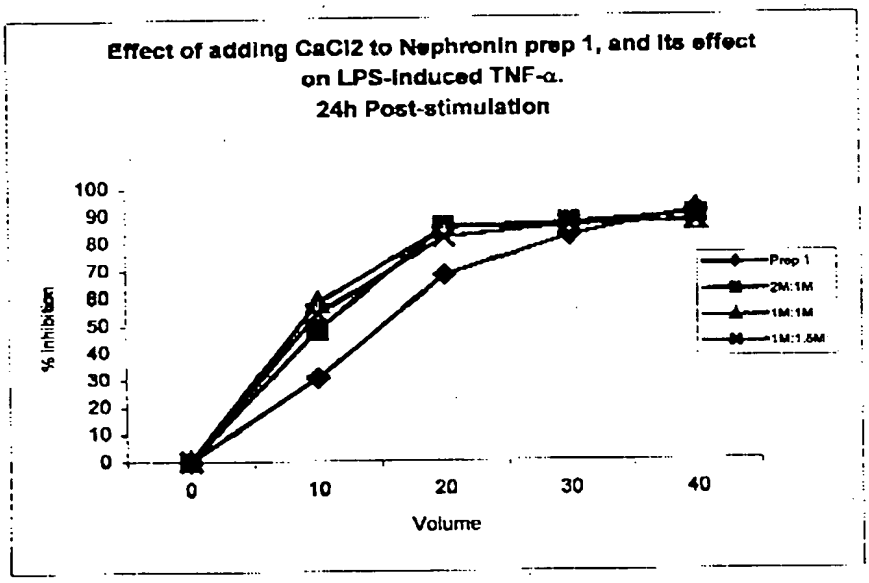
Fig 6



Increasing concentrations of LPS were used to stimulate PBMC. 40μl of each preparation was used in the inhibition of LPS-induced TNF-α production.
In each case 40μl of each preparation was added to chambers of the tissue culture plates containing LPS that would give the indicated concentration in 1ml.
1 ml of PBMC at $1.5x10^6$ cells/ml was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 1



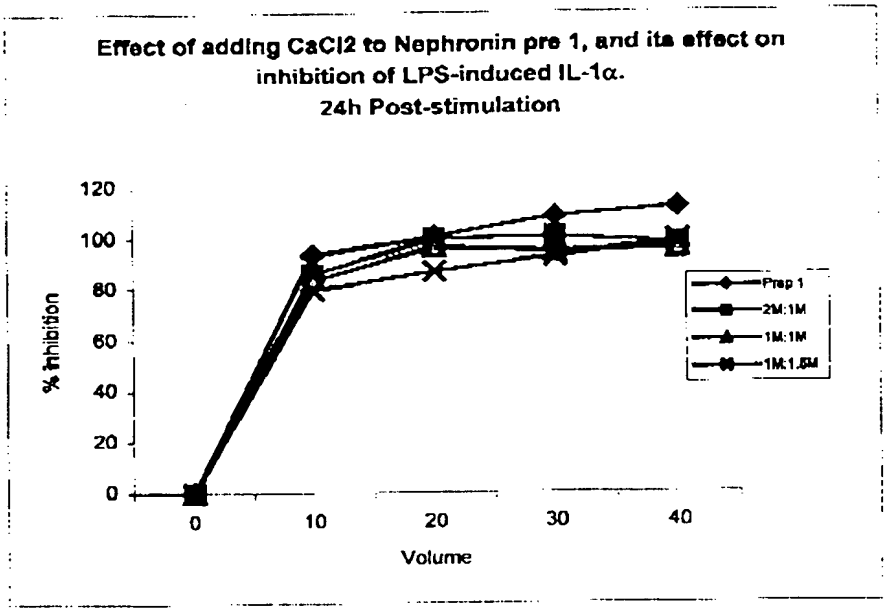
Comparison of dose dependent inhibition profile for LPS-induced TNF-α production by Nephronin, preparation 1 from experiment 23, treated with CaCl2.
2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.

1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1.5M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1.5M of CaCl2 was added.
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 2



Comparison of dose dependent inhibition profile for LPS-induced IL-1α production by Nephronin, preparation 1 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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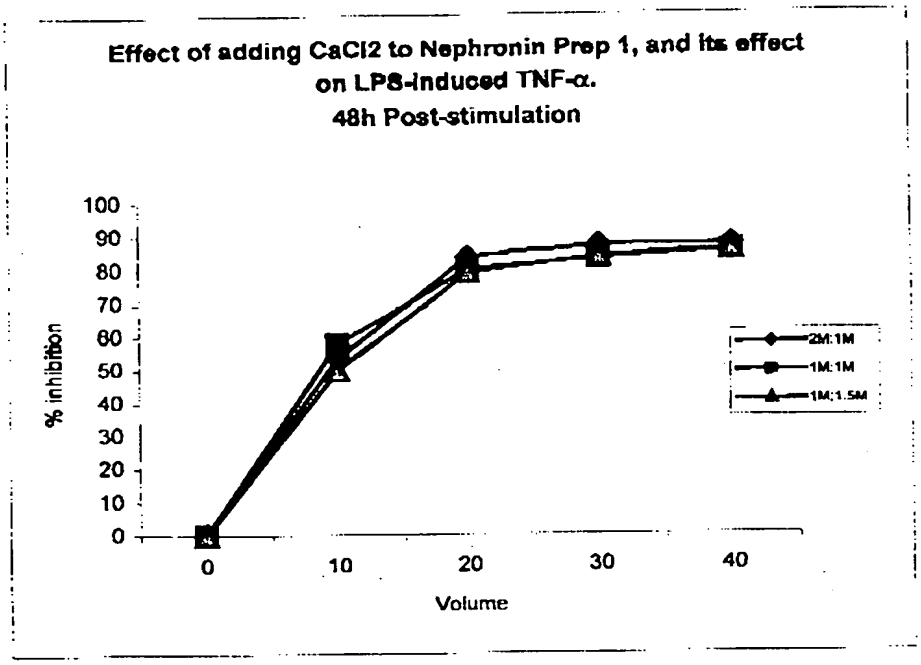
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA.

Experiment 24

Fig 3



Comparison of dose dependent inhibition profile for LPS-induced TNF-α production by Nephronin, preparation 1 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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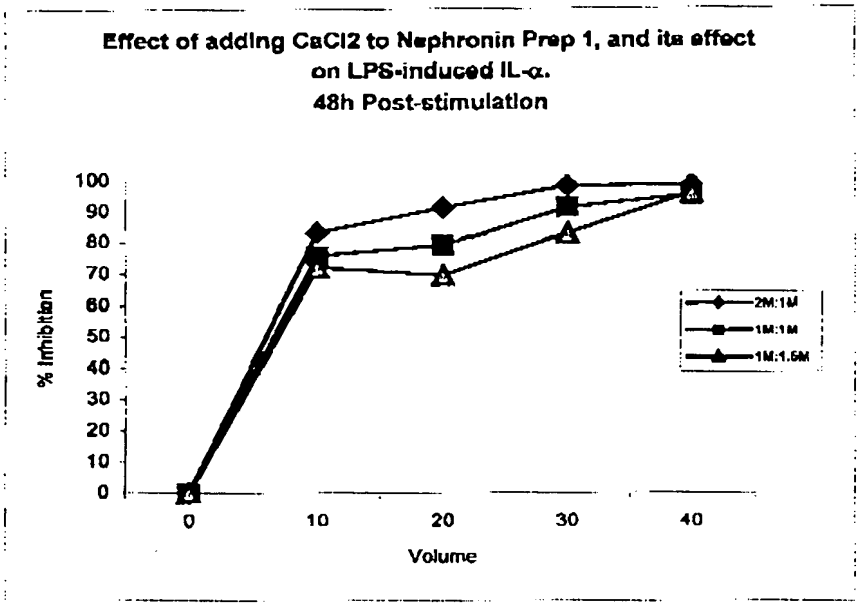
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 48h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 4



Comparison of dose dependent inhibition profile for LPS-induced IL-1α production by Nephronin, preparation 1 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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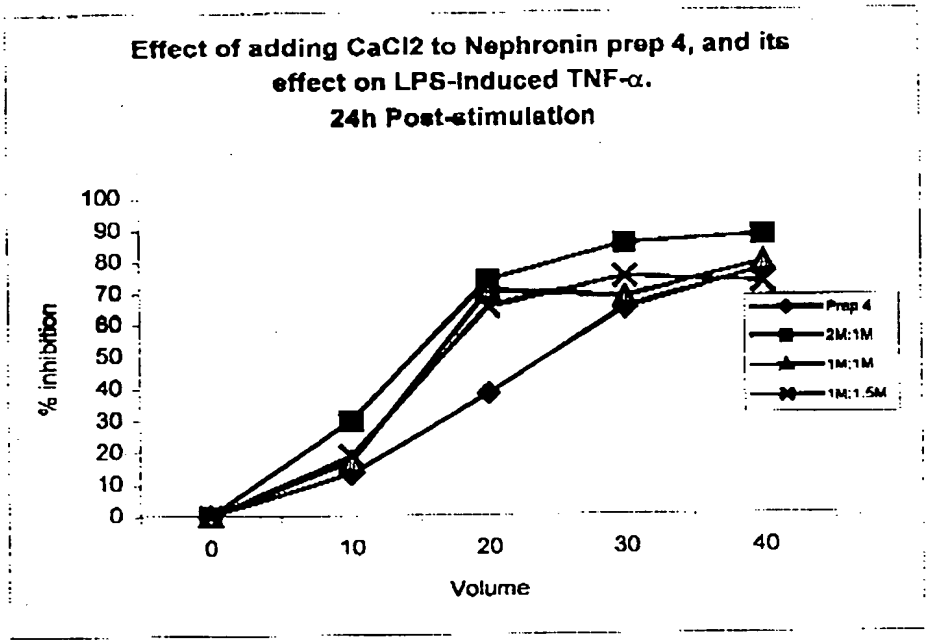
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 48h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA.

Experiment 24

Fig 5



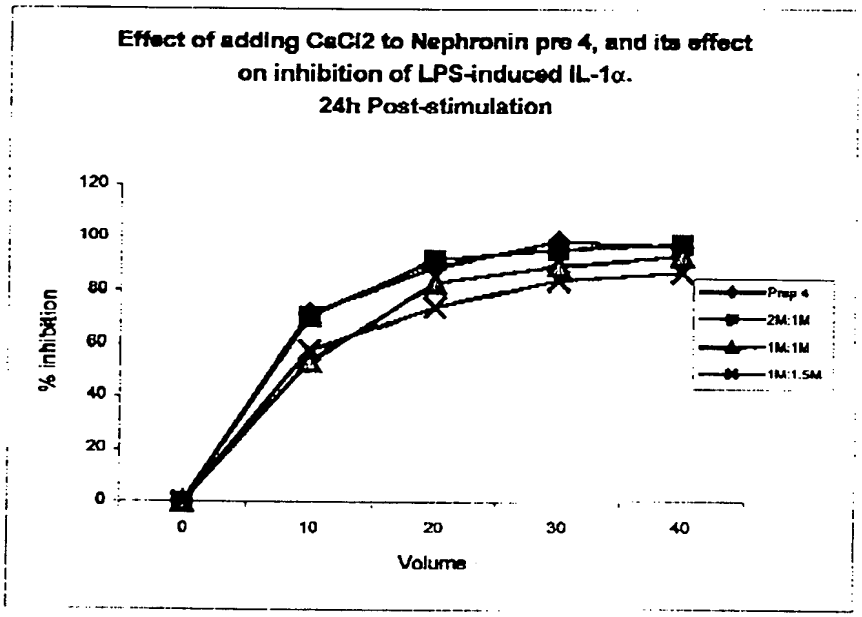
Comparison of dose dependent inhibition profile for LPS-induced TNF-α production by Nephronin, preparation 4 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1.5M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1.5M of CaCl2 was added.
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 6



Comparison of dose dependent inhibition profile for LPS-induced IL-1α production by Nephronin, preparation 4 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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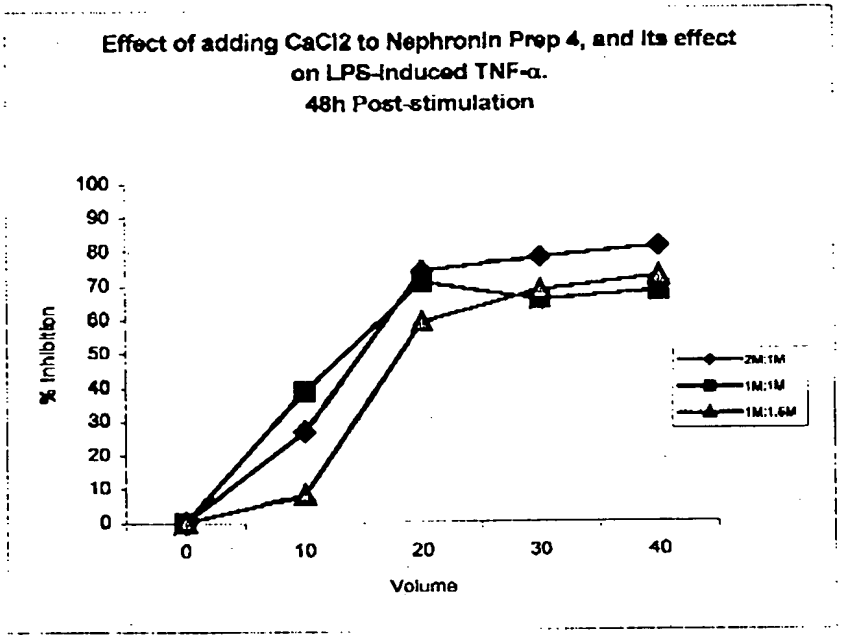
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA.

Experiment 24

Fig 7



Comparison of dose dependent inhibition profile for LPS-induced TNF-α production by Nephronin, preparation 4 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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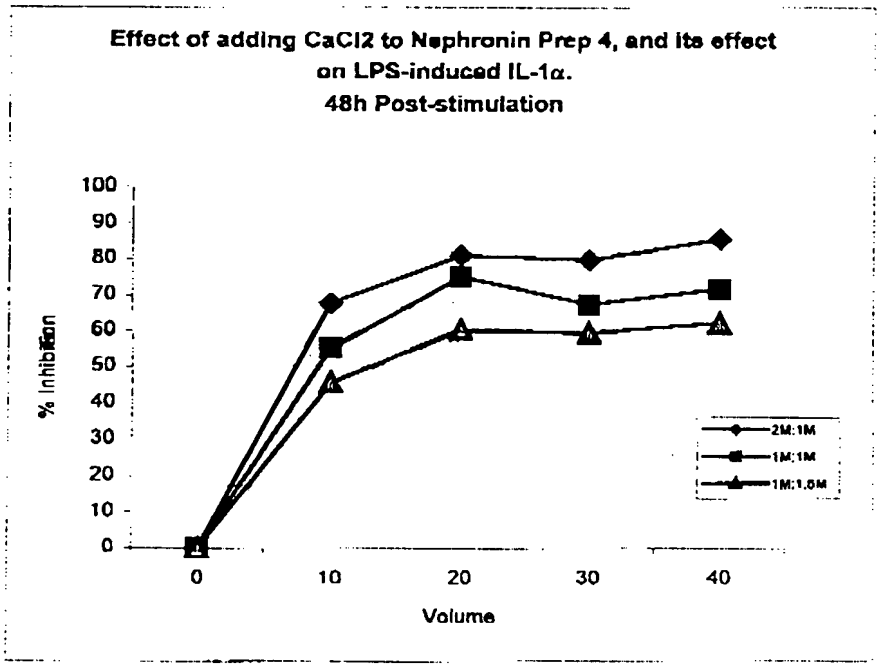
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 48h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 8



Comparison of dose dependent inhibition profile for LPS-induced IL-1α production by Nephronin, preparation 4 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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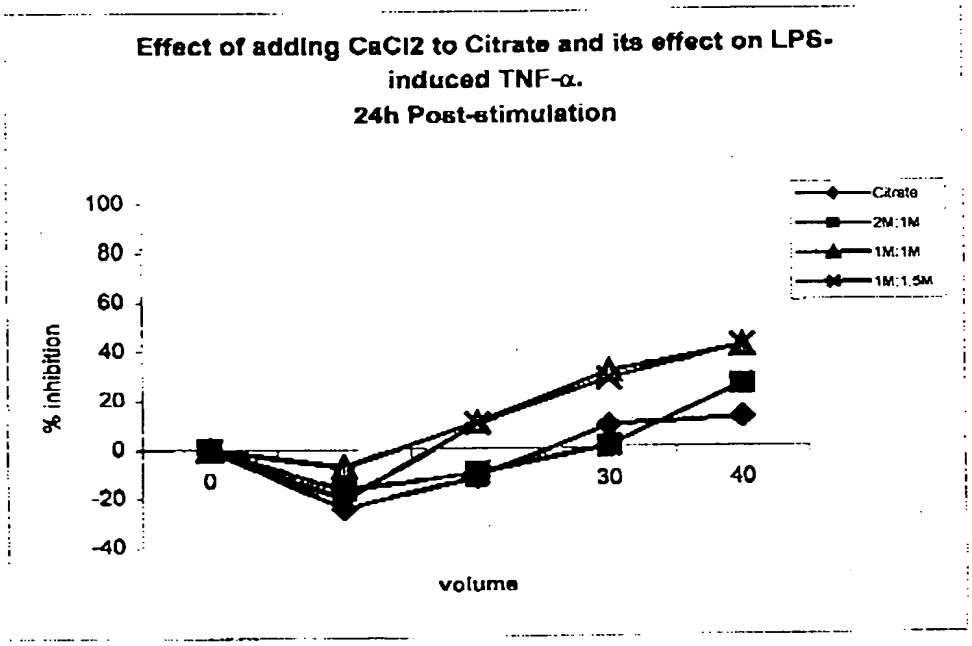
Experiment 24

Fig 9



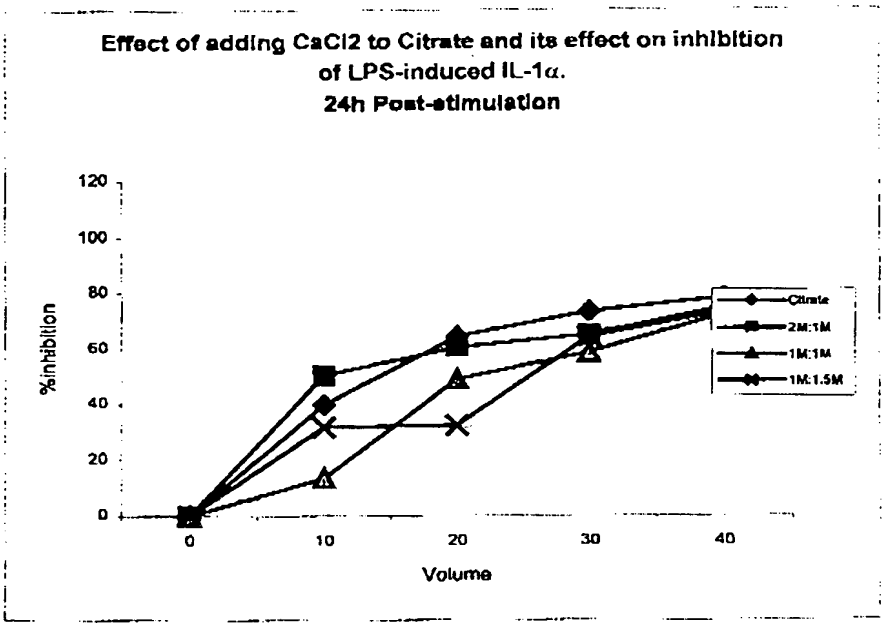
Comparison of dose dependent inhibition profile for LPS-induced TNF-α production by a solution of 0.25M ammonium citrate, pH 7.4.

2M:1M refers to treated ammonium Citrate solution where for every 2M of ammonium Citrate 1M of CaCl2 was added.
1M:1M refers to treated ammonium Citrate solution where for every 1M of ammonium Citrate 1M of CaCl2 was added.
1M:1.5M refers to treated ammonium Citrate solution where for every 1M of ammonium Citrate 1.5M of CaCl2 was added.
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 10



Comparison of dose dependent inhibition profile for LPS-induced IL-1α production by a solution of 0.25M ammonium citrate, pH 7.4.

2M:1M refers to treated ammonium Citrate solution where for every 2M of ammonium Citrate 1M of CaCl2 was added.
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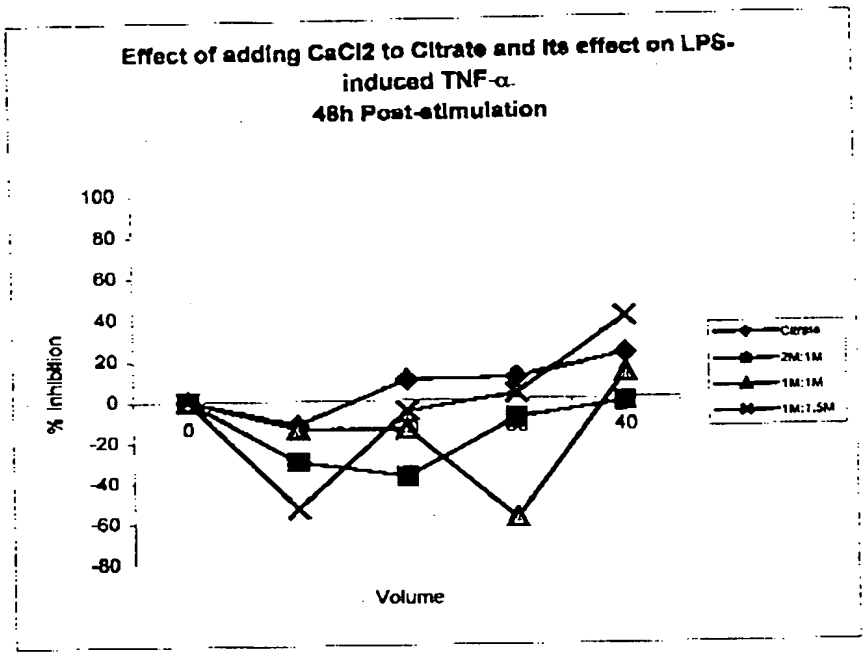
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 24h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA.

Experiment 24

Fig 11



Comparison of dose dependent inhibition profile for LPS-induced TNF-α production by a solution of 0.25M ammonium citrate, pH 7.4.

2M:1M refers to treated ammonium Citrate solution where for every 2M of ammonium Citrate 1M of CaCl2 was added.
1M:1M refers to treated ammonium Citrate solution where for every 1M of ammonium Citrate 1M of CaCl2 was added.
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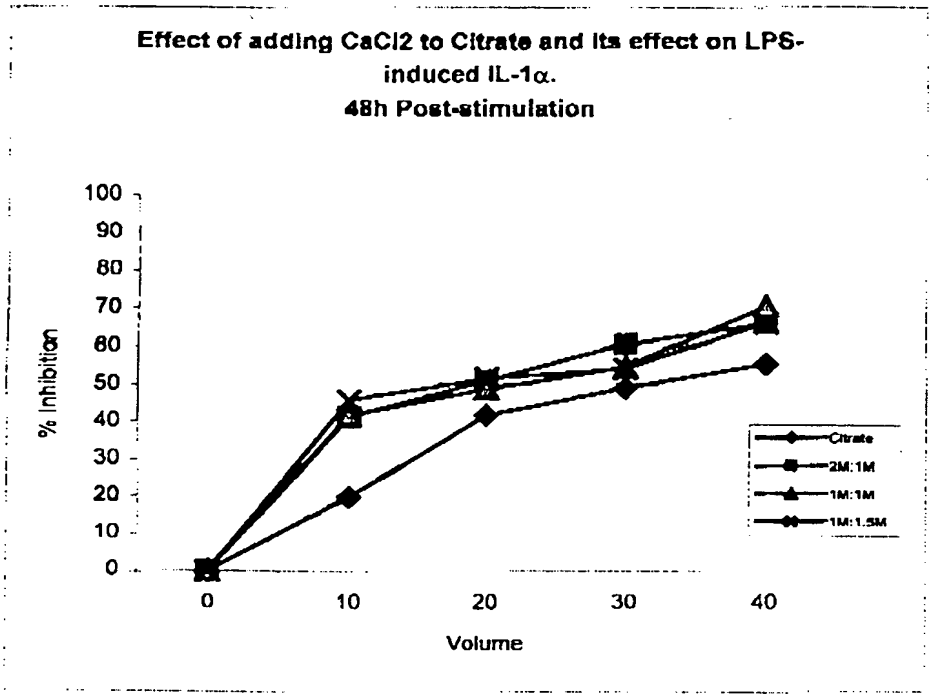
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at 1.5×10^6 cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 48h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 12



Comparison of dose dependent inhibition profile for LPS-induced IL-1α production by a solution of 0.25M ammonium citrate, pH 7.4.

2M:1M refers to treated ammonium Citrate solution where for every 2M of ammonium Citrate 1M of CaCl2 was added.
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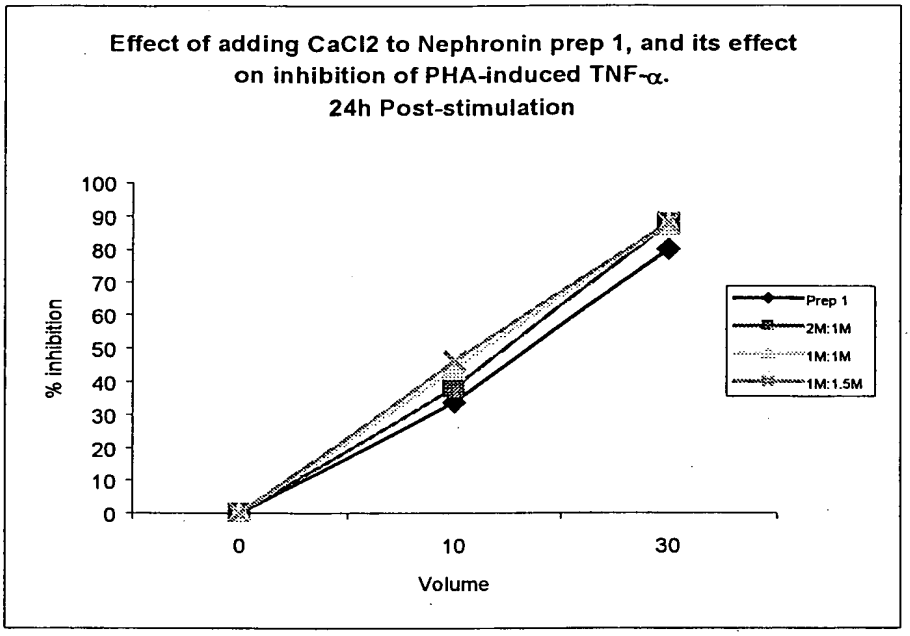
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates. 1 ml of PBMC at $1.5x10^6$ cells/ml containing 1ng/ml of LPS was added to each well. The cells were harvested 48h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA.

Experiment 24

Fig 13



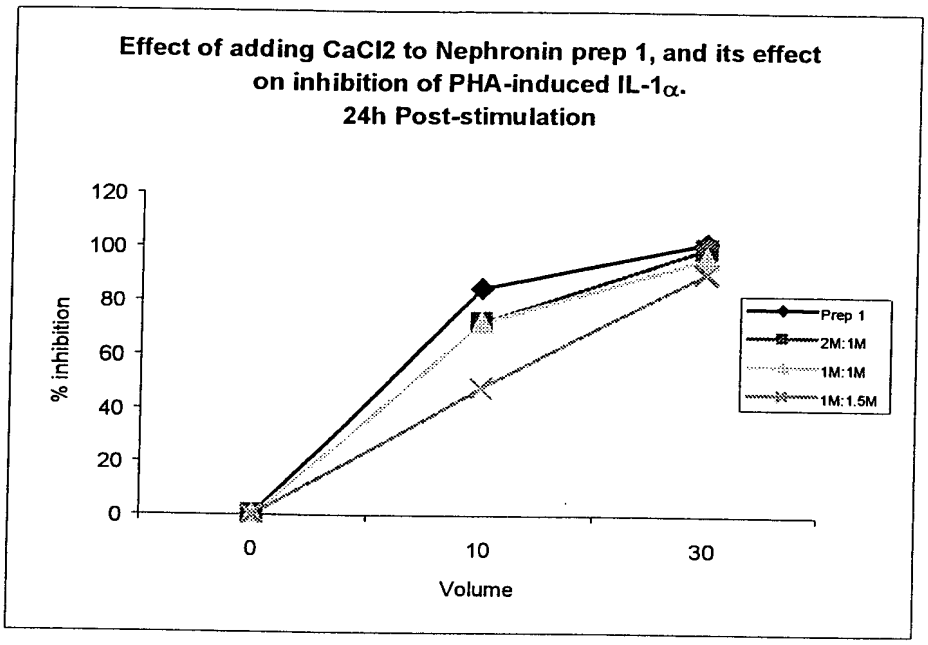
Comparison of dose dependent inhibition profile for PHA-induced TNF-α production by Nephronin, preparation 1 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1.5M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1.5M of CaCl2 was added.
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates containing 5μg of PHA. 1 ml of PBMC at 1.5×10^6 cells/ml was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 14



Comparison of dose dependent inhibition profile for PHA-induced IL-1α production by Nephronin, preparation 1 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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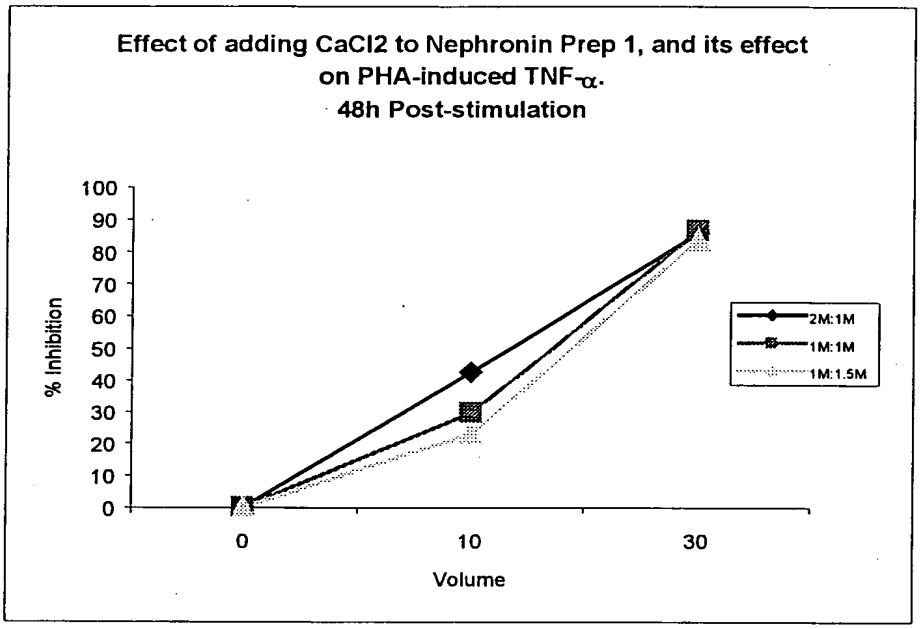
Experiment 24

Fig 15



Comparison of dose dependent inhibition profile for PHA-induced TNF-α production by Nephronin, preparation 1 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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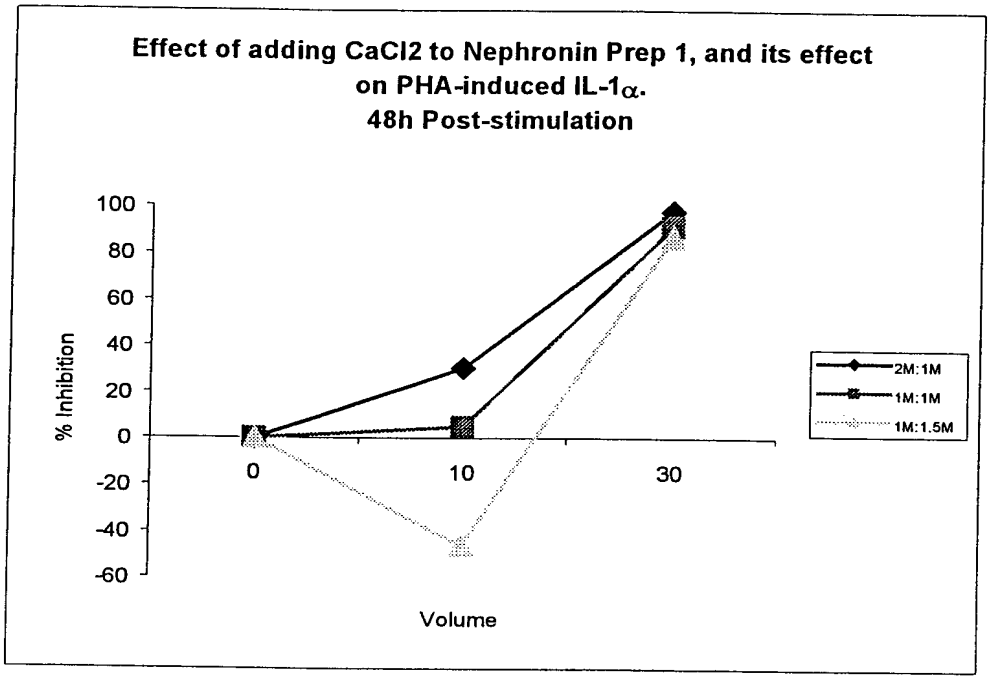
In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates containing 5μg of PHA. 1 ml of PBMC at $1.5x10^6$ cells/ml was then added to each well. The cells were harvested 48h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 16



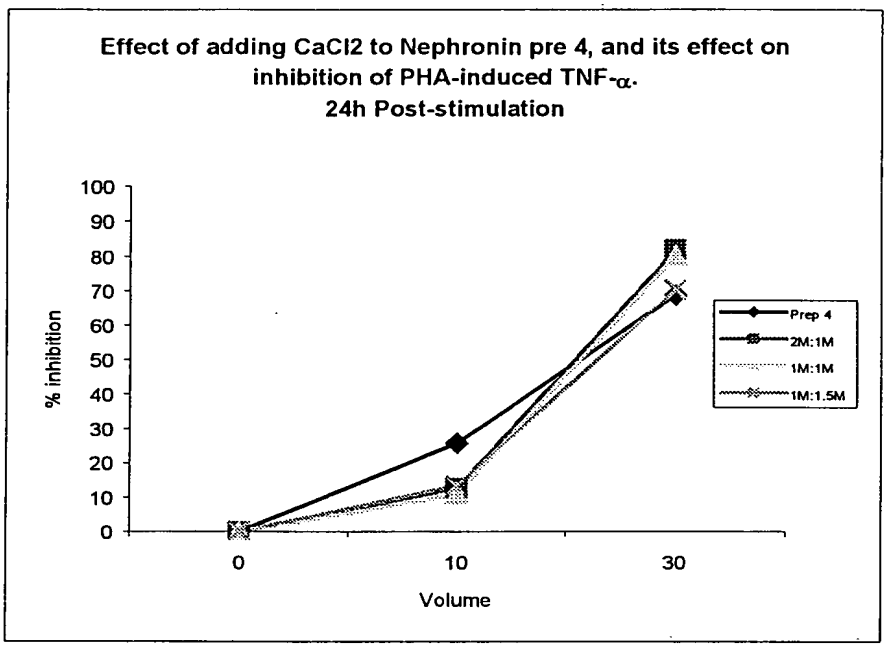
Comparison of dose dependent inhibition profile for PHA-induced IL-1α production by Nephronin, preparation 1 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1.5M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1.5M of CaCl2 was added.
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates containing 5μg of PHA. 1 ml of PBMC at 1.5×10^6 cells/ml was then added to each well. The cells were harvested 48h post-stimulation and the concentration of IL-1α was determined by sandwich ELISA.

Experiment 24

Fig 17



Comparison of dose dependent inhibition profile for PHA-induced TNF-α production by Nephronin, preparation 4 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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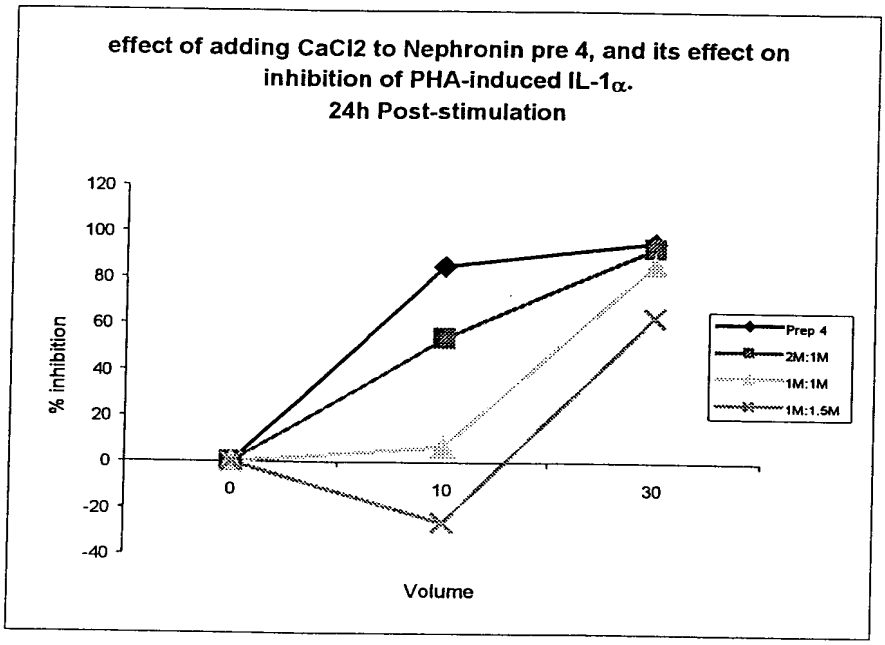
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates containing 5μg of PHA. 1 ml of PBMC at $1.5x10^6$ cells/ml was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 18



Comparison of dose dependent inhibition profile for PHA-induced IL-1α production by Nephronin, preparation 4 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
1M:1M refers to treatment of Nephronin where for every 1M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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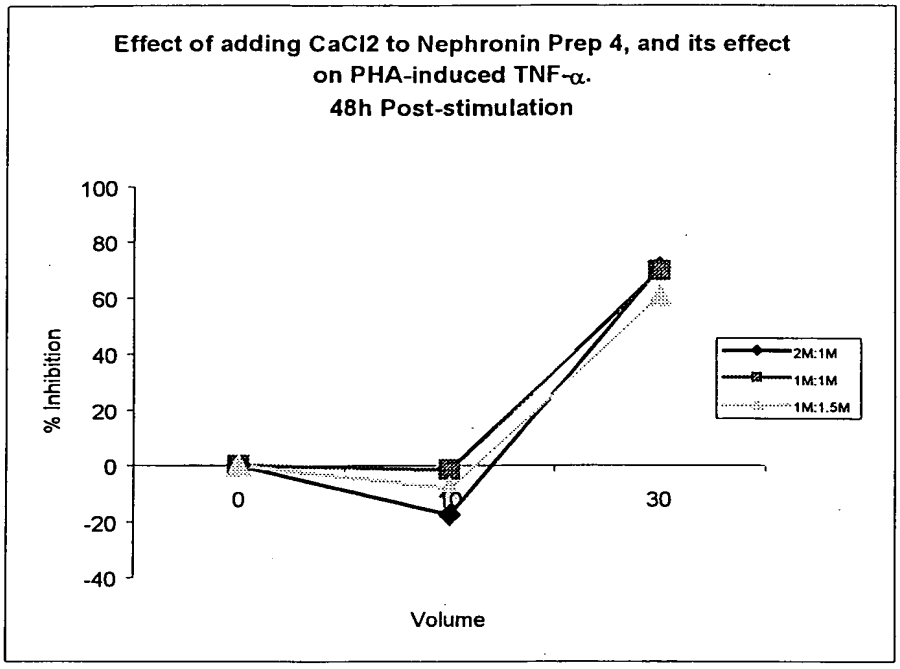
Experiment 24

Fig 19



Comparison of dose dependent inhibition profile for PHA-induced TNF-α production by Nephronin, preparation 4 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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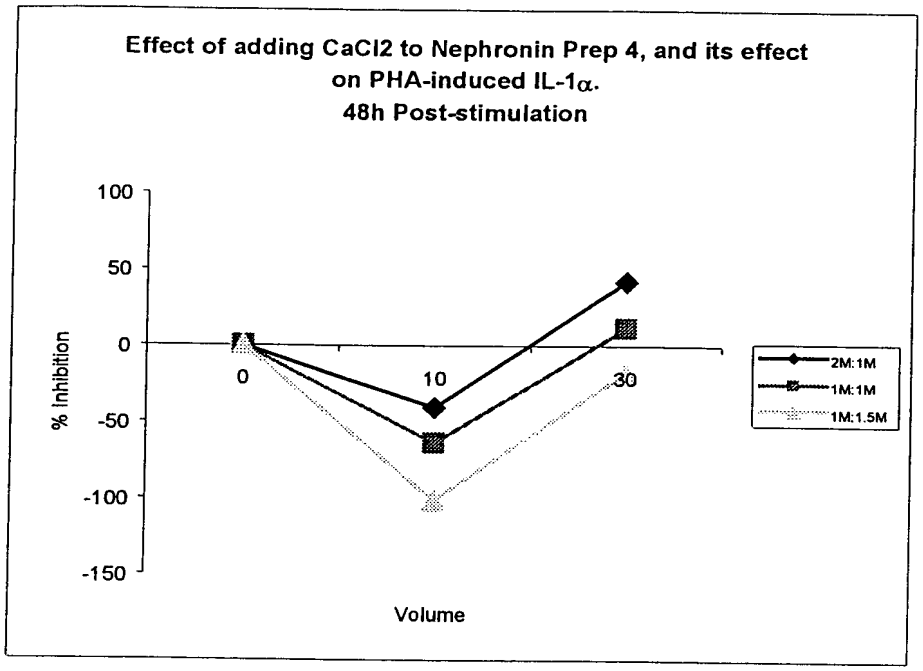
Experiment 24

Fig 20



Comparison of dose dependent inhibition profile for PHA-induced IL-1α production by Nephronin, preparation 4 from experiment 23, treated with CaCl2.

2M:1M refers to treatment of Nephronin where for every 2M of Na3Citrate in the preparation, 1M of CaCl2 was added.
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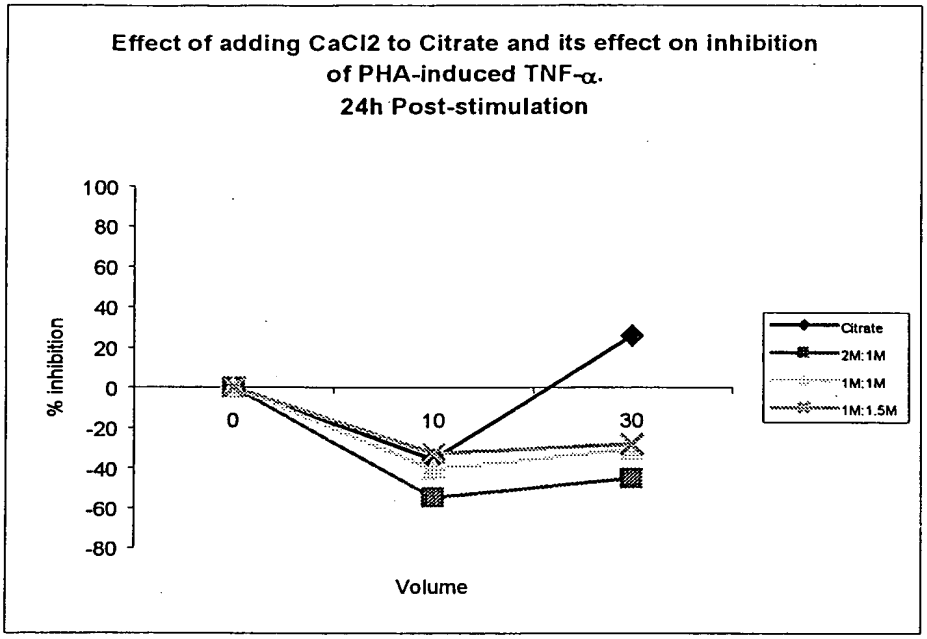
Experiment 24

Fig 21



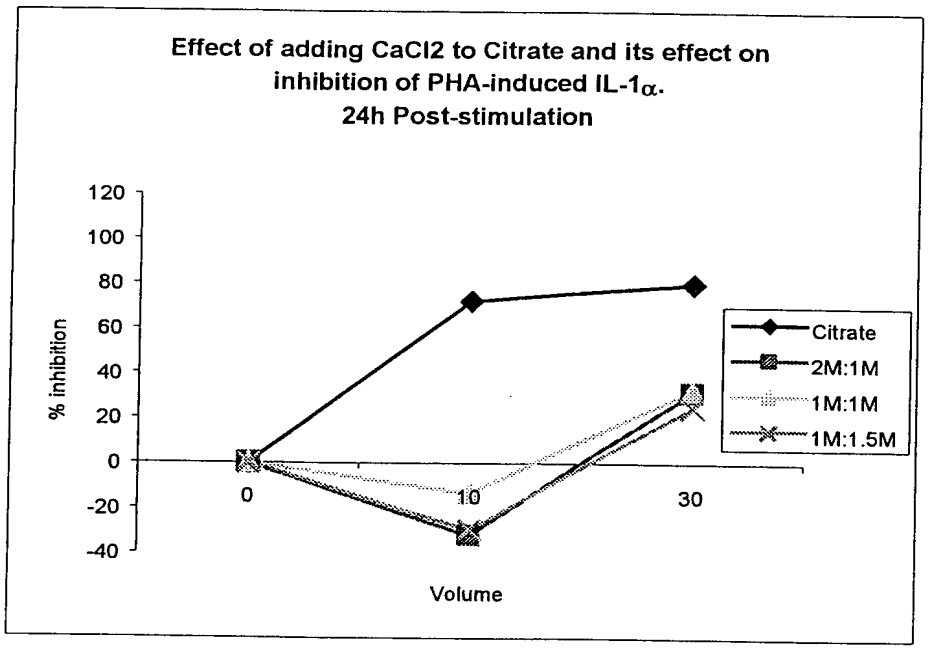
Comparison of dose dependent inhibition profile for PHA-induced TNF-α production by a solution of 0.25M ammonium citrate, pH 7.4.

2M:1M refers to treated ammonium Citrate solution where for every 2M of ammonium Citrate 1M of CaCl2 was added.
1M:1M refers to treated ammonium Citrate solution where for every 1M of ammonium Citrate 1M of CaCl2 was added.
1M:1.5M refers to treated ammonium Citrate solution where for every 1M of ammonium Citrate 1.5M of CaCl2 was added.
The samples were mixed and the resulting precipitates were removed by centrifugation.

In each case, the indicated volume of each sample was added to chambers of 24 well tissue culture plates containing 5μg of PHA. 1 ml of PBMC at $1.5x10^6$ cells/ml was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.s

Experiment 24

Fig 22



Comparison of dose dependent inhibition profile for PHA-induced IL-1α production by a solution of 0.25M ammonium citrate, pH 7.4.

2M:1M refers to treated ammonium Citrate solution where for every 2M of ammonium Citrate 1M of CaCl2 was added.
1M:1M refers to treated ammonium Citrate solution where for every 1M of ammonium Citrate 1M of CaCl2 was added.
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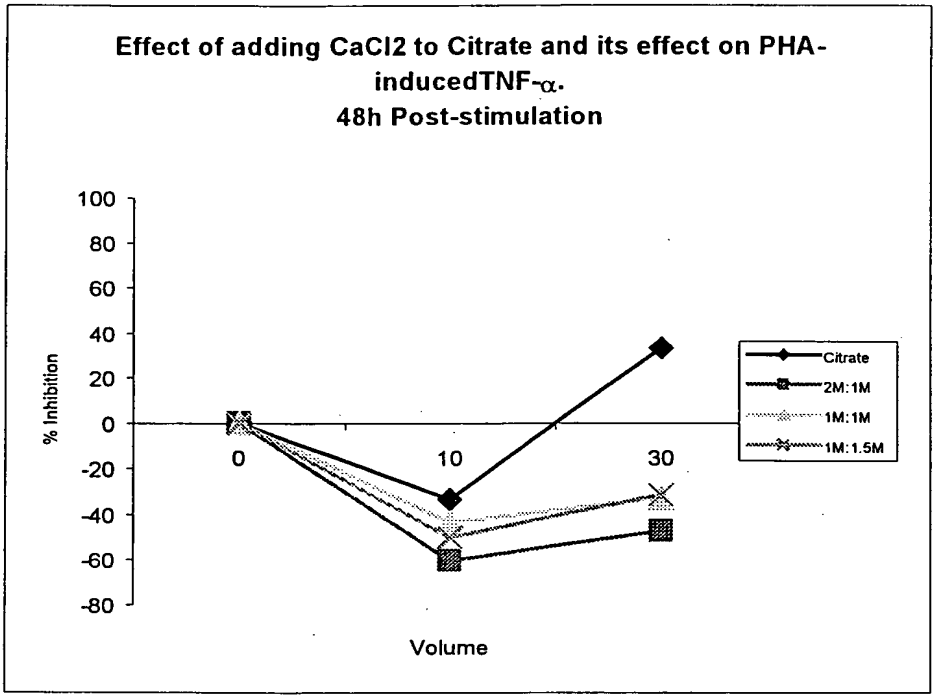
Experiment 24

Fig 23



Comparison of dose dependent inhibition profile for PHA-induced TNF-α production by a solution of 0.25M ammonium citrate, pH 7.4.

2M:1M refers to treated ammonium Citrate solution where for every 2M of ammonium Citrate 1M of CaCl2 was added.
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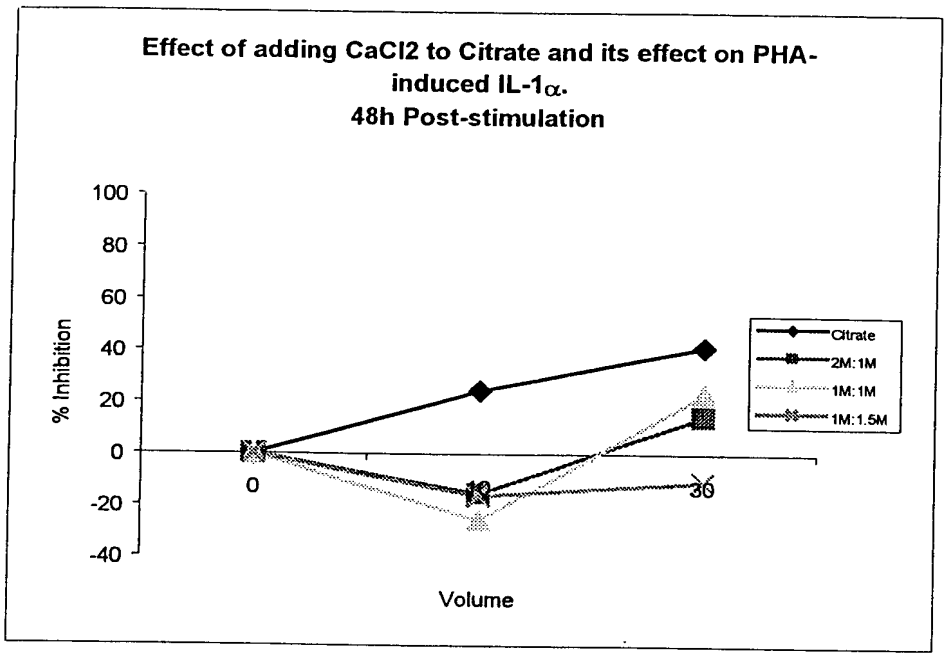
Experiment 24

Fig 24



Comparison of dose dependent inhibition profile for PHA-induced IL-1α production by a solution of 0.25M ammonium citrate, pH 7.4.

2M:1M refers to treated ammonium Citrate solution where for every 2M of ammonium Citrate 1M of CaCl2 was added.
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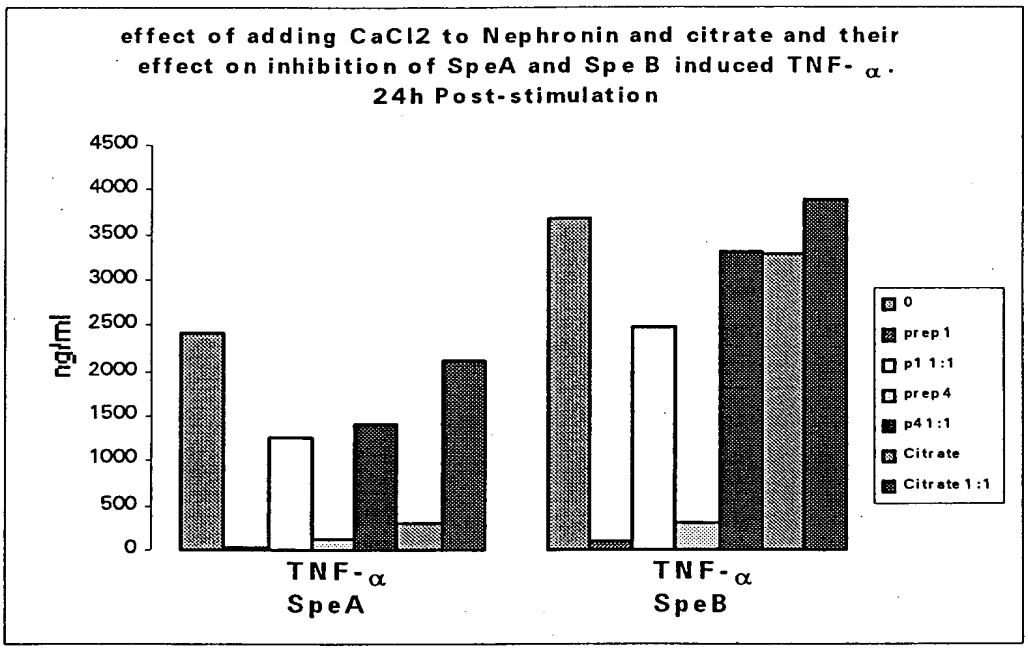
Experiment 24

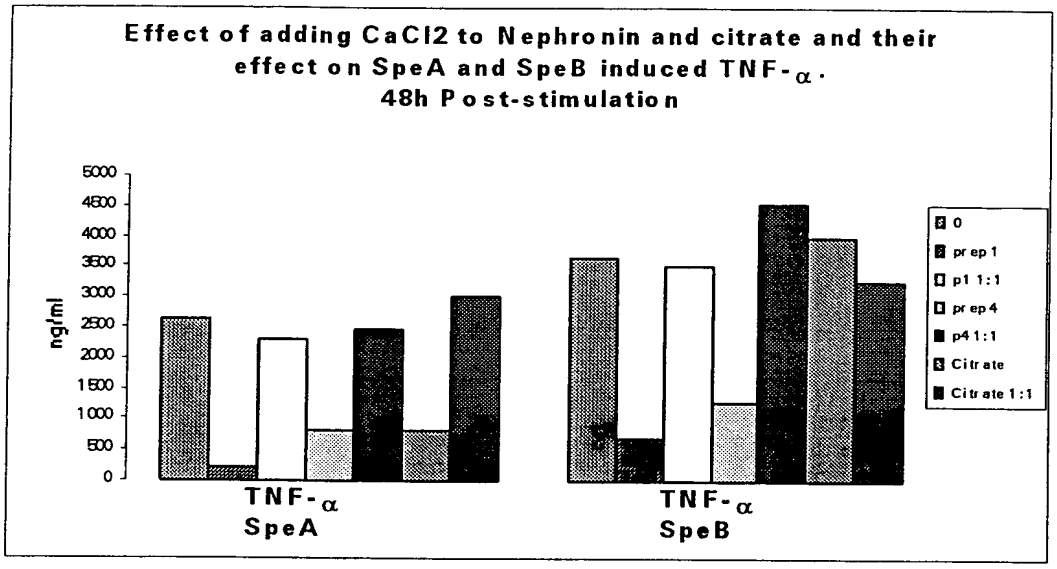
Fig 25



Comparison of inhibitory effects of two preparations of Nephronin preps 1 and 4, from experiment 23 and 0.25M ammonium citrate. The effect of the same samples treated with 1M:1M ratio of CaCl2 on the basis of concentration of Na3Citrate in the Nephronin preparations.
50ng of SpeA and SpeB were added to the appropriate chambers of the tissue culture plates. 10µl of the preparations was added to the chambers. 1 ml of PBMC at $1.5x10^6$ cells/ml was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 26



Comparison of inhibitory effects of two preparations of Nephronin preps 1 and 4, from experiment 23 and 0.25M ammonium citrate. The effect of the same samples treated with 1M:1M ratio of CaCl2 on the basis of concentration of Na3Citrate in the Nephronin preparations.

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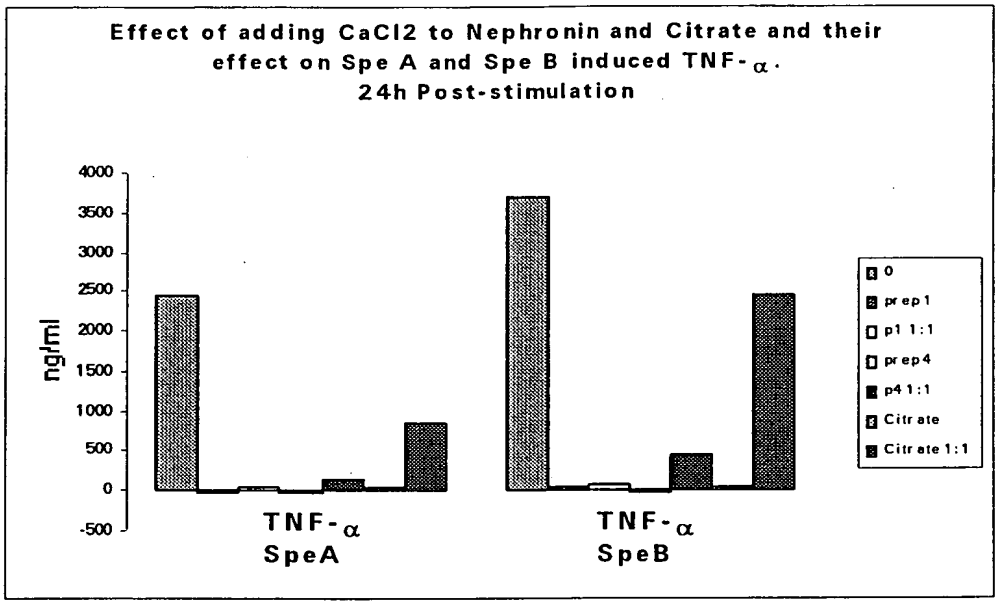
Experiment 24

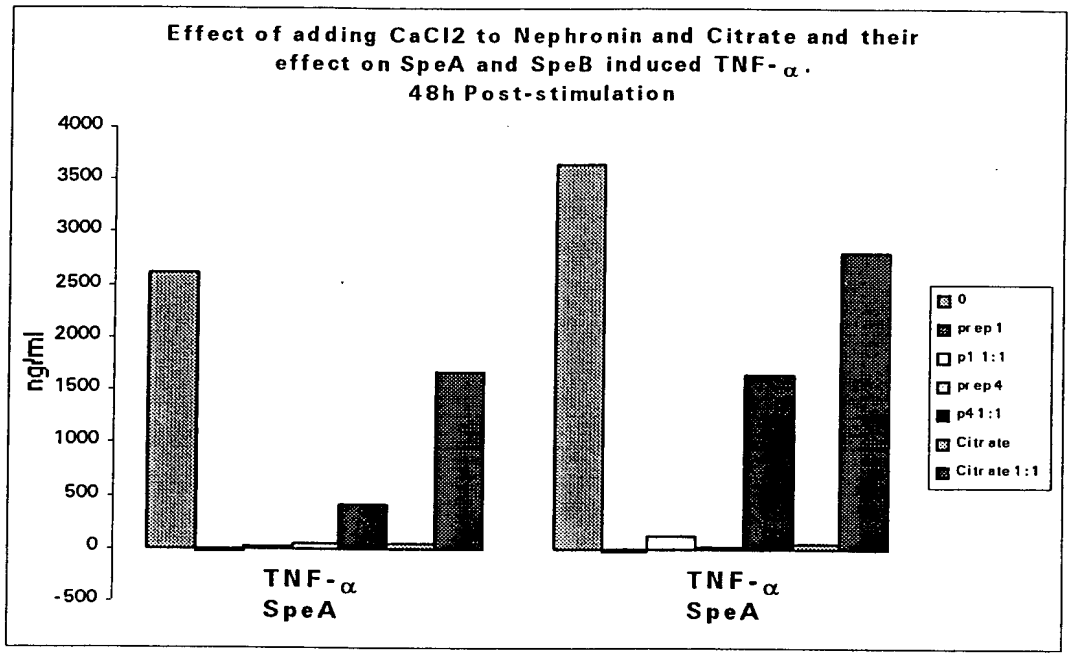
Fig 27



Comparison of inhibitory effects of two preparations of Nephronin preps 1 and 4, from experiment 23 and 0.25M ammonium citrate. The effect of the same samples treated with 1M:1M ratio of CaCl2 on the basis of concentration of Na3Citrate in the Nephronin preparations.
50ng of SpeA and SpeB were added to the appropriate chambers of the tissue culture plates. 30µl of the preparations was added to the chambers. 1 ml of PBMC at $1.5x10^6$ cells/ml was then added to each well. The cells were harvested 24h post-stimulation and the concentration of TNF-α was determined by sandwich ELISA.

Experiment 24

Fig 28



Comparison of inhibitory effects of two preparations of Nephronin preps 1 and 4, from experiment 23 and 0.25M ammonium citrate. The effect of the same samples treated with 1M:1M ratio of CaCl2 on the basis of concentration of Na3Citrate in the Nephronin preparations.

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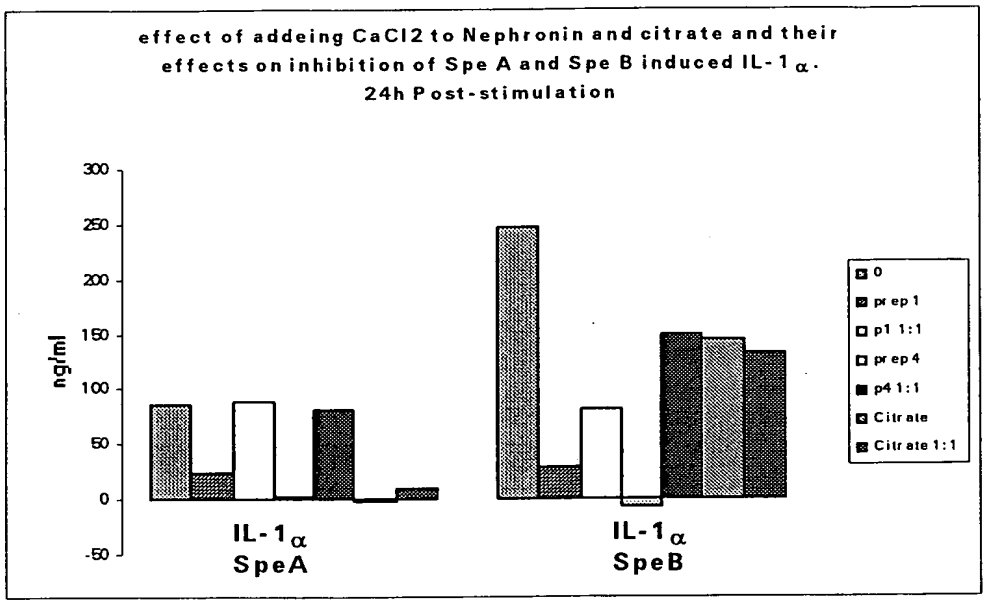
Experiment 24

Fig 29



Comparison of inhibitory effects of two preparations of Nephronin preps 1 and 4, from experiment 23 and 0.25M ammonium citrate. The effect of the same samples treated with 1M:1M ratio of CaCl2 on the basis of concentration of Na3Citrate in the Nephronin preparations.

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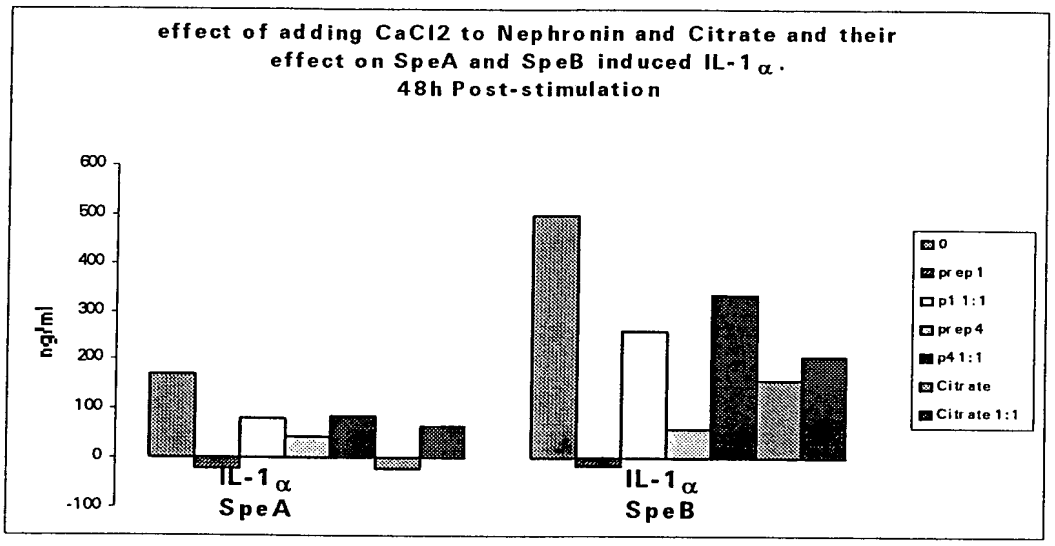
Experiment 24

Fig 30



Comparison of inhibitory effects of two preparations of Nephronin preps 1 and 4, from experiment 23 and 0.25M ammonium citrate. The effect of the same samples treated with 1M:1M ratio of CaCl2 on the basis of concentration of Na3Citrate in the Nephronin preparations.
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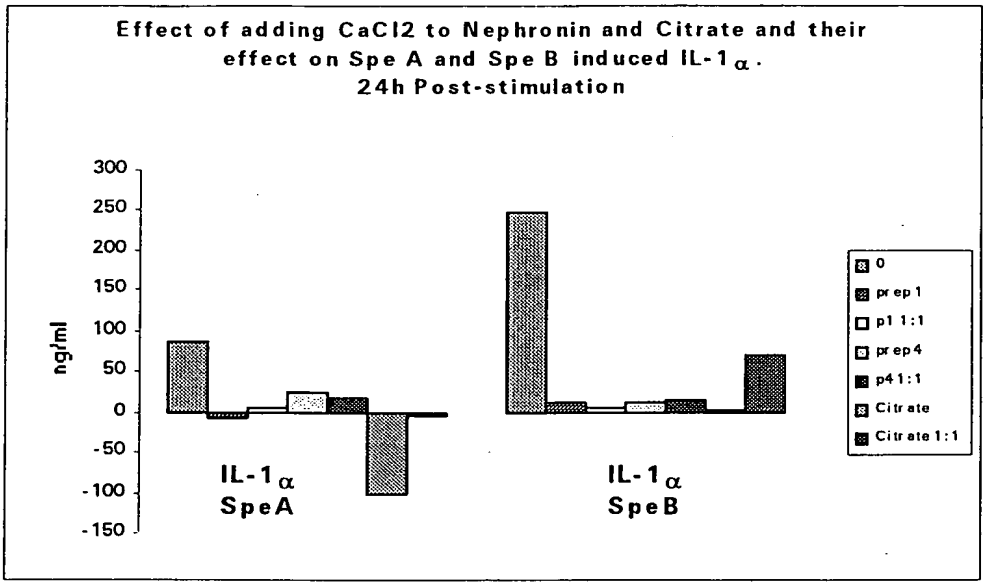
Experiment 24

Fig 31



Comparison of inhibitory effects of two preparations of Nephronin preps 1 and 4, from experiment 23 and 0.25M ammonium citrate. The effect of the same samples treated with 1M:1M ratio of CaCl2 on the basis of concentration of Na3Citrate in the Nephronin preparations.

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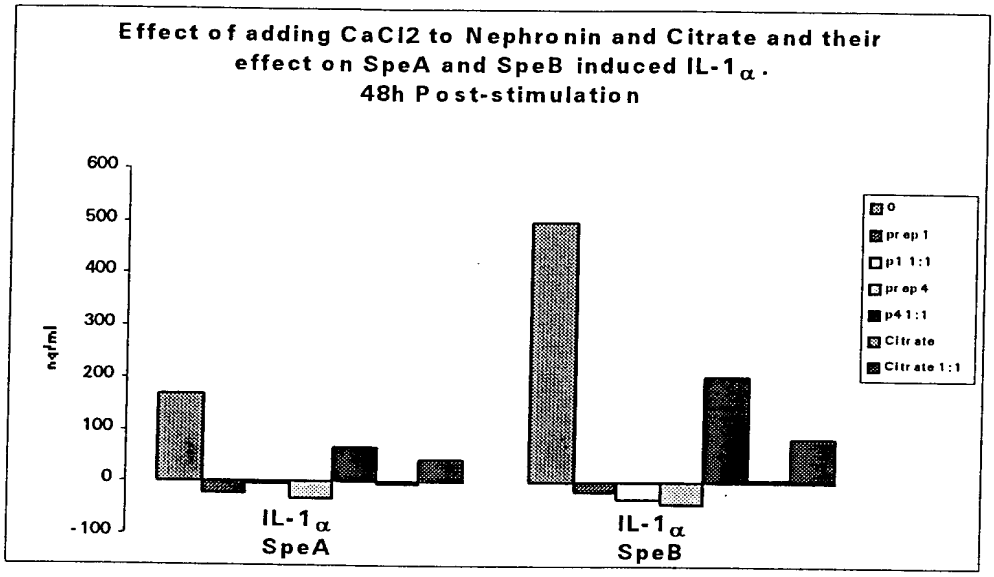
Experiment 24

Fig 32



Comparison of inhibitory effects of two preparations of Nephronin preps 1 and 4, from experiment 23 and 0.25M ammonium citrate. The effect of the same samples treated with 1M:1M ratio of CaCl2 on the basis of concentration of Na3Citrate in the Nephronin preparations.

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